REMARKS

Applicant would like to thank the Examiner for confirming that the instant official action is a non-final action, in a telephonic communication with Jeanne M. Brashear (Reg. No. 56,30~~1)~~ ~~o~~n April 13, 2005.

I. THE CLAIMS ARE IN FULL COMPLIANCE WITH 35 U.S.C. § 101

On pages 2-11 of the Office action, the Office rejected claims 156-219 under 35 U.S.C. § 101 for allegedly not being supported by either a specific and substantial utility or a well-established utility. Applicant respectfully traverses and requests that the rejection be withdrawn as improper for the reasons discussed herein.

A. The Claims are Supported by a Specific, Substantial, and Credible Utility as Well as a Well-Established Utility as Set Forth in the Specification

The claimed invention has utility in accordance with both 35 U.S.C. § 101 and the Office's utility guidelines as it possesses a well-established utility as well as a specific, substantial and credible utility.

1. The Claimed Invention Has A Well-Established Utility

The claimed invention comprises a collectin and as such it possesses a well-established utility. The identification of the invention as a collectin is sufficient to establish utility under 35 U.S.C. § 101. Beginning on page 1 of the specification, the claimed polypeptide and polynucleotide is identified as a collectin, and the known, established properties of collectins are described, *e.g.*, anti-bacterial and anti-viral activity. *See, e.g.*, page 1, line 9, to page 2, line 16. Figure 5 shows the alignment of the claimed polypeptide with known collectins, see page 3, lines 23-25. Furthermore, the claimed polypeptide shares 100% identity over 547 amino acids with collectin, CL-P1 (collectin placenta-1). CL-P1 is described in further detail below.

The Examiner questions whether the 35% sequence similarity to collectin SP-D is enough similarity to predict function. In reality, there is a great variation in percent identity among members of the collectin family. For example, collectins SP-D and CL-L1 (collectin liver-1) have only 29% identity over 264 amino acids; collectins MBP-A and SP-A have only 35% identity over 254 amino acids; and collectins SP-A and SP-D have only 40% identity over 235 amino acids. The claimed polypeptide has 35% identity with SP-D over

302 amino acids. This percent identity is commensurate with other known members of the collectin family. As such, the similarity supports applicant's position that the molecule is a collectin. Moreover, the record contains important additional evidence, such as domain structure of the molecule and also its calcium-dependent carbohydrate binding activity, as discussed below.

As confirmation of Applicant's asserted utility, a rule 132 declaration was provided in the response filed on August 6, 2004 in which the inventor described experiments that confirmed the claimed invention has utility as set forth in the specification. The inventor showed that the claimed polypeptide is able to bind to various saccharides (carbohydrates) in a calcium-dependent manner, a property consistent with C-type lectins, including collectins. More particularly, the claimed polypeptide was able to bind galactose in a calcium-dependent manner. The collectin polypeptide was prepared and tested in its ability to bind various saccharides in the relative presence and absence of calcium. The results demonstrated that depletion of calcium ions with EDTA prevents the binding between the polypeptide and the saccharides tested. Accordingly the data discussed by the inventor confirmed that the claimed polypeptide comprises a collectin and possesses utilities associated with the same, and the rejection should be withdrawn.

Several of the publications of record also support the designation of the claimed subject matter as a collectin. For example, Hoppe and Reid (Ref. C30; Protein Science 3:1143-58 (1994)), describe the common structure of collectins (at pages 1144-1149), carbohydrate recognition (at pages 1149-1150) and binding of collectins to bacteria and viruses (at pages 1150-1152), and the Hoppe description is consistent with the collectin claimed in the present application and described in the specification.

The art accepts that the CL-P1 is a collectin even though it has a transmembrane domain, in contrast to the classical collectins which are soluble and secreted proteins. Van de Wetering et al. (Exhibit A, Eur. J. Biochem., 271:1230-1249, 2004) describe members of the collectin family and their functions in innate immunity. One of the collectin family members, CL-P1 (collectin placenta-1), shares 100% identity to the claimed polypeptide sequence over 547 amino acids. CL-P1 is a membrane-bound collectin that is involved in the uptake of oxidized LDL particles. It has also been determined that CL-P1 recognizes *E. coli* and *S. aureus* (Exhibit B, Lu et al., Biochim. Biophys. Acta, 1572:387-400,

2000). Of note is the fact that CL-P1 preferentially binds galactose over mannose. The same binding properties are found in the claimed polypeptide sequence (see Rule 132 declaration, page 4). The art accepts that the full length CL-P1 sequence functions as a collectin. The smaller molecule claimed in the instant application has an active domain that is sufficient to maintain galactose binding in a calcium-dependent manner, and thereby preserves a well-established utility of a collectin.

The fact that the claimed protein is a member of the collectin family is further evidenced by acceptance of the inventor's publication in a prominent journal. Ohtani et al., (Exhibit C, J. Biol. Chem. 276:44222-44228, 2001) describe a collectin, CL-P1, that can also function as a scavenger receptor. Ohtani further describes the ability of CL-P1 to form an oligomeric structure and its ability to bind and induce phagocytosis of bacteria and yeast as well as oxidized LDL. The fact that the prominent journal, Journal of Biological Chemistry, accepted and published this manuscript is evidence that scientists in the field would regard the molecule as a collectin.

Accordingly, because the claimed subject matter is a collectin and collectins have a well-established utility, the objection is improper and should be withdrawn.

2. The Claimed Invention Has A Specific, Substantial, and Credible Utility

The claimed invention has both a specific and substantial utility, and this utility is credible. To have a specific utility, the claimed species, *e.g.*, a protein, possesses a utility that is specific, *i.e.*, one not common to all proteins. The claimed species comprises a protein that binds saccharides in a calcium dependent manner. More particularly, the claimed polypeptide binds glalactose in a calcium dependent manner. Not all proteins have such characteristics, which means the claimed species has a specific utility. The claimed species is also substantial. To be substantial, the claimed subject matter must have a "real world" use. Standing alone, calcium-dependent galactose binding makes the claimed protein useful for affinity purifying galactose and galactose-containing complex structures, and then releasing the purified structures with changes in calcium. Also, the claimed collectin has utility as an anti-viral compound in the inhibition of infection. *See* pages 1 and 2 of the specification. To be credible, the utility must be believable to a person of ordinary skill in the art based on the totality of the evidence and the reasoning provided. The evidence and the

reasoning provided establish that the claimed subject matter comprises a collectin and functions as such (see above discussion of the § 1.132 declaration as well as the support in literature of record). Accordingly, the rejection is improper and should be withdrawn.

II. THE CLAIMS FIND SUFFICIENT WRITTEN DESCRIPTIVE SUPPORT IN THE SPECIFICATION

A. The Specification Teaches How To Use The Claimed Invention

On page 11 of the Office action, the Office rejected claims 156-219 under 35 U.S.C. § 112, first paragraph, as the invention was allegedly not supported by either a specific or substantial asserted utility or a well-established utility for the reasons described in relation to the § 101 rejection. Applicant respectfully traverses and submits that the rejection should be withdrawn for the same reasons as those described above in section I.

B. The Claimed Subject Matter Is Adequately Described In The Specification

On page 12 of the Office action, the Office rejected various claims under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter that was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. Applicant respectfully traverses and submits that the rejection should be withdrawn.

Written description support for the hybridization conditions set forth in the claims can be found on page 5, lines 1-11, of the application as filed.

Accordingly, the claims have proper written descriptive support in the specification as filed, the rejection is improper, and the rejection should be withdrawn.

CONCLUSION

For the foregoing reasons, the Applicant respectfully submits that claims 156-219 are in condition for allowance. The Office is invited to contact the undersigned at the telephone number listed below in order to discuss any remaining issues or matters of form will move this case to allowance. A check covering the necessary fees is enclosed, and the attached transmittal forms authorize the Office to charge any additional fees to Applicant's account.

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Respectfully submitted,

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EXHIBIT A

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REVIEW ARTICLE

Collectins

Players of the innate immune system

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Collectins are a family of collagenous calcium-dependent defense lectins in animals. Their polypeptide chains consist of four regions: a cysteine-rich N-terminal domain, a collagen-like region, an α-helical coiled-coil neck domain and a C-terminal lectin or carbohydrate-recognition domain. These polypeptide chains form trimers that may assemble into larger oligomers. The best studied family members are the mannan-binding lectin, which is secreted into the blood by the liver, and the surfactant proteins A and D, which are secreted into the pulmonary alveolar and airway lining fluid. The collectins represent an important group of pattern recognition molecules, which bind to oligosaccharide structures and/or lipid moities on the surface of microorganisms. They bind preferentially to monosaccharide units of the mannose type, which present two vicinal hydroxyl groups in an equatorial position. High-affinity interactions between collectins and microorganisms depend, on the one hand, on the high density of the carbohydrate ligands on the microbial surface, and on the other, on the degree of oligomerization of the collectin. Apart from binding to microorganisms, the collectins can interact with receptors on host cells. Binding of collectins to microorganisms may facilitate microbial clearance through aggregation, complement activation, opsonization and activation of phagocytosis, and inhibition of microbial growth. In addition, the collectins can modulate inflammatory and allergic responses, affect apoptotic cell clearance and modulate the adaptive immune system.

Keywords: collectin, C-type lectin; mannan-binding lectin (MBL); surfactant protein A (SP-A); surfactant protein D (SP-D); innate immunity; host defense; surface epitopes; pulmonary surfactant; infectious disease.

Introduction

Collectins belong to the super family of mammalian C-type lectins, and are believed to be involved in innate defense systems. The following eight collectins have been identified so far: mannan-binding lectin (MBL), surfactant protein A (SP-A), surfactant protein D (SP-D), collectin liver 1 (CL-L1), collectin placenta 1 (CL-P1), conglutinin, collectin of 43 kDa (CL-43) and collectin of 46 kDa (CL-46). As part of the innate immune system, collectins have a key role in the first line of defense against invading microorganisms, as demonstrated by elegant experiments with genetically manipulated mice made deficient in MBL, SP-A or SP-D, which show increased susceptibility to bacterial and viral infections. Apart from CL-L1 and CL-P1, which are found in the cytosol and cell membrane, respectively, all collectins are soluble and secreted proteins. An important property of the collectins is their capability to recognize pathogen-associated molecular patterns on foreign organisms, which involves distinguishing between self and nonself carbohydrate structures. This review gives an overview of what is currently known about the functions of the collectins in host defense, the sites of their production, and their structure and function. Emphasis will be on the molecular basis of their recognition of carbohydrate structures.

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Abbreviations: CL-43, collectin of 43 kDa; CL-46, collectin of 46 kDa; CL-L1, collectin liver 1; CL-P1, collectin placenta 1; CRD, carbohydrate recognition domain; HA, hemagglutinin; HSV-1, herpes simplex virus type 1; IAV, influenza A virus; IFN-γ, interferon-γ; IL, interleukin; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MASP, MBL-associated serine protease; MBL, mannan-binding lectin; RSV, respiratory syncytial virus; SIRPα, signal regulating protein α; SP-A, surfactant protein A; SP-D, surfactant protein D; TLR, toll-like receptor; TNF-α, tumor necrosis factor-α.

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Sites of collectin production

MBL is secreted into the bloodstream, and is mainly produced by the liver [1–3]. In rodents [4,5], rabbits [6,7], and rhesus monkeys [8] two forms of MBL have been found (MBL-A and MBL-C), whereas in humans and chimpanzees only one form was shown to be present [8]. Although the liver is the main site of MBL-A and MBL-C production in mice, mRNA expression has been detected in various tissues. However, substantial expression of *MBL-A* and *MBL-C* was only demonstrated in the kidney and small intestine, respectively, where expression could also be demonstrated at the protein level using immunohistochemistry [9,10]. The presence of substantial amounts of protein in the small intestine suggests that

MBL acts as a humoral immune factor in the intestine, similar to secretory IgA.

The lung collectins SP-A and SP-D were first shown to be present in the alveolar space of the lung, and it has long been established that alveolar type II cells [11–13] and nonciliated bronchial epithelial cells (Clara cells) [13,14] are the major sites of synthesis. Although the major site of SP-A and SP-D synthesis is the lung, both lung collectins have been detected in extrapulmonary tissues as well. Using RT-PCR, low amounts of *SP-A* mRNA have been shown to be present in a number of murine tissues, whereas on the protein level there were only indications for the presence of SP-A in the murine uterus [15]. In addition to its presence in the murine uterus, low levels of SP-A have also been detected in the porcine eustachian tube [16]. Whereas extrapulmonary SP-A expression seems to be limited to a few organs, SP-D has been detected in many nonpulmonary tissues, on the mRNA as well as protein level, and tissue distribution was found to depend on the animal species studied [15–17].

Using Northern blot analysis, high levels of *CL-L1* mRNA were found in the liver and a weaker signal was demonstrated in the placenta. RT-PCR revealed the presence of low copy numbers of *CL-L1* mRNA in most tissues except for skeletal tissue. Although most collectins are secreted, CL-L1 was only detected in the cytosol of hepatocytes, suggesting that this protein might react with intracellular ligands [18]. CL-P1 was detected in vascular endothelial cells, while *CL-P1* mRNA could be demonstrated in many tissues. This is the only collectin identified so far that is membrane bound, and contains an intracellular domain [19].

The serum collectins conglutinin, CL-46 and CL-43 have so far only be detected in bovidae, where the liver is their main site of production [20]. The reason for the presence of this wide array of serum collectins in bovidae is unknown but might be related to the fact that these animals live in symbiosis with an enormous amount of microbes in their rumen. One could speculate that the bovine serum collectins provide a first line of defense against these microbes, when they leak into the bloodstream, without eliciting a general inflammatory reaction involving antibodies, which might be detrimental to the fine host-microbial balance in their rumen. It would be of interest to see whether bovidae are the only ruminants that express these additional serum collectins, and in addition, whether nonruminant herbivores like horses, which rely heavily on the microbial symbiosis in their large appendix, have similar collectin-based serum defense mechanisms.

Protein levels of both SP-A and SP-D in the alveolar compartment increase in response to pulmonary infection with microorganisms [21], and SP-D levels increase in allergen-induced eosinophilia [22], indicating that both proteins might function as analogues of acute phase reactants in the lung. Interestingly, hyperoxia also induces an increase in SP-A and SP-D concentrations in the alveolar compartment [23]. As damaged epithelium is more susceptible to infection, this might represent a mechanism by which oxygen-damaged alveolar epithelium protects itself against the increased susceptibility to invading microorganisms.

The recent demonstration of MBL [9,10], SP-A [15,16,24] and SP-D [15–17,24,25] expression at mucosal surfaces suggests that these proteins have a general function in innate immunity at these locations and more specifically in the gastrointestinal tract. In addition, the finding that SP-D expression in the gastric mucosa is significantly increased during *Helicobacter pylori* infection, further points to the possibility of SP-D having a role in mucosal defense systems outside the lung [25].

Structure of the collectins

The basic functional unit of collectins is a trimer. The number of trimeric units per collectin molecule differs among the collectins. In the monomeric subunits, four structural domains can be distinguished: an N-terminal cysteine-rich domain, a collagen domain, a coiled-coil neck domain and finally a C-type lectin domain, also known as carbohydrate recognition domain (CRD) (Fig. 1).

The CRDs of collectins are compactly folded protein modules of 115–130 amino acid residues and are located at the C-terminus of the protein [26]. Selective binding of collectins to specific complex carbohydrates is mediated by their CRDs, and requires the presence of calcium [26,27]. The actual carbohydrate binding site can be found in a shallow groove in the CRD [27–29].

Comparison of the CRD domains of soluble collectins has revealed that 22 amino acids are conserved within this domain. Most of these conserved residues, including four cysteine residues, that form intrachain disulfide bridges, are involved in proper folding of the CRD [30]. CRDs contain several calcium binding sites, although the exact number of ligated calcium ions under physiological conditions is as yet not totally clear. Crystallographic analysis showed the presence of three and two calcium ions in the CRD of rat MBL-A and MBL-C, respectively [27,28], whereas MBL-A

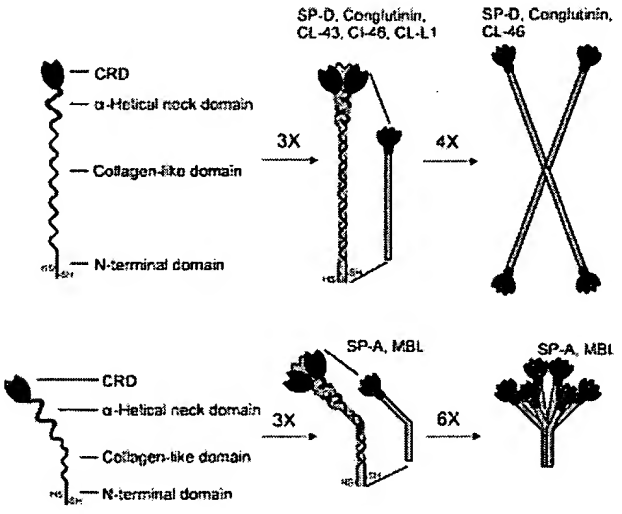

Fig. 1. Schematic representation of the domain organization and tertiary structures of the collectins. The carbohydrate recognition domain (CRD) is followed by an α-helical neck domain, a collagen-like domain and an N-terminal cysteine (SH)-rich domain. Three neck domains will form a triple coiled-coil structure, and the collagen-like domain will assemble into a triple helix, leading to the formation of trimeric subunits. Trimeric subunits are assembled subsequently via cysteine residues in the N-terminal domain into higher oligomeric forms.

Table 1. Effects of the degree of oligomerization and truncation of SP-D on various of its activities. $(CRD)_1$, monomeric CRD; $(CRD)_3$, trimeric CRD/neck domain with or without N-terminus of SP-D; $(SP\text{-}D)_3$, trimeric SP-D; $(SP\text{-}D)_{12}$, dodecameric SP-D; $(SP\text{-}D)_m$, multimeric SP-D. Where no – or + symbols are given, no data are available. Column $[(SP\text{-}D)_{12}/(SP\text{-}D)_m]$ shows reports in which no distinction was made between dodecameric and multimeric SP-D. Higher magnitude of activity is indicated by a greater number of + symbols.

Activity of SP-D	$(CRD)_1$	$(CRD/neck)_3$	$(SP\text{-}D)_3$	$(SP\text{-}D)_{12}$	$[(SP\text{-}D)_{12}/(SP\text{-}D)_m]$	$(SP\text{-}D)_m$	Refs.
Binding to (poly)saccharides	–	+	+ +	+ + +	+ + +		[33,35,36]
Inhibition of hemagglutination by IAV		+	+ +	+ + +		+ + + +	[37–39]
Aggregation of IAV		–	+/–	+		+ +	[37–39]
IAV binding to neutrophils		–	–	+		+ +	[37–39]
Enhancement of IAV-induced respiratory burst of neutrophils		–	+/–	+		+ +	[37–39]
Protection against neutrophil inactivation by IAV		–	+/–	+		+ +	[37–39]
Aggregation of *E. coli*			–	+		+ +	[40]
Stimulation of phagocytosis of *E. coli*				+		+ +	[40]
Inhibition of phagocytosis of *M. tuberculosis*		+		+			[41]
Stimulation of chemotaxis		+			+ +		[42]
Protection against *A. fumigatus*-induced allergy		+[a]			+		[43]

[a] Homotrimers consisting of CRD/neck and eight Gly-Xaa-Yaa repeats from the collagen region.

binding data indicated the presence of only two calcium ions per CRD [26]. It has been suggested that the third calcium ion found in the MBL-A CRD crystal resulted from the excess of calcium in the crystallization buffer (15 mM) [27]. However, it was demonstrated that, although it was crystallized in the presence of only 1 mM calcium, the crystal of the SP-D CRD also contained three calcium ions [29]. Moreover, crystallization of the recombinant homotrimeric fragment of SP-D, comprising the CRD and α-helical neck domain, in the presence of about 2.5 mM calcium, but in the absence of saccharide ligand, even revealed the presence of a fourth calcium ion. This latter calcium ion was found to be present in the funnel formed by the three CRDs and close to the neck–CRD interface [31]. Although several calcium ions are present within the CRDs of collectins, monosaccharide binding by their CRDs occurs through direct coordination of one of the calcium ions and hydrogen bond interactions with side-chains of amino acids that also serve as ligands for this calcium ion [27–29,32]. The observation that the α–helical neck domain of SP-D on its own may bind to LPS and phospholipids, and that this interaction is calcium dependent [33], suggests that the fourth calcium ion found in SP-D might be involved in ligand interactions as well.

The exact function of each of the two calcium ions – found in the CRD away from the neck region – that are not involved directly in ligand interactions is not exactly known, but there are indications that at least one of them is involved in the correct folding of the CRD in order to allow carbohydrate binding [31,34]. Shrive *et al.* [31] hypothesized that the calcium ions not involved directly in monosaccharide binding may be involved in binding more extended ligands, or that they are involved in the recognition of immune cell surface receptors. In addition, the electrostatic potential pattern on the surface of the protein might be altered by the additional calcium ions, thereby influencing the affinity for negatively charged ligands.

As indicated above, collectins are multimeric proteins. The degree of multimerization can greatly affect their function. This has been extensively studied for SP-D. The effects of the degree of oligomerization on various functions of this protein (which will be discussed later in this review) are given in Table 1. For the first step in the oligomerization of the collectins, the trimerization of monomers, the presence of the coiled-coil neck domain is essential [32,33, 44–47]. Recombinant proteins consisting only of the neck and CRD region are still assembled as trimers, whereas isolated CRDs lacking the neck domain are secreted as monomers [33,48], or in the case of MBL, as dimers [27]. Recently, it was demonstrated that specific heptad repeats within the hydrophobic neck domain are required for the formation of stable trimeric SP-D subunits. It is thought that the primary role of the neck domain in molecular assembly is to align the collagen chains and thereby facilitate subsequent 'zipper-like' folding of the collagen helix [45].

The collagen-like region of the collectins consists of repeating motifs of Gly-X-Y, where X and Y can be any amino acid, but frequently are proline or hydroxyproline. The collagen helices of monomers are coiled around each other, to form a stable tensile collagen domain that is relatively resistant to proteases [49,50]. Another interesting structural feature of the collagen domain is that it can be N-glycosylated or O-glycosylated [49,50]. The repeat Gly-X-Y pattern in both MBL and SP-A is interrupted, which is thought to introduce a kink or region of flexibility into the protein, enabling the trimeric subunits to angle away from the central core, to form a structure resembling a bouquet of flowers [51,52] (Fig. 1).

The collagen domain of collectins is thought to have several (distinct) functions. It has been shown for SP-A and MBL that the collagen domain is involved in receptor-mediated effects of both proteins [53,54]. A specific GEKGEP motif within the collagen domain of MBL was shown to be involved in binding to the C1q receptor [54]. Interestingly, the amino acid sequence of the collagen

domain of SP-A contains a similar motif [55] that might also be involved in the demonstrated interaction of SP-A with the C1q receptor [56–58]. SP-D, which does not interact with the C1q receptor, does not contain this motif [59,60]. The collagen domain of MBL is also involved in the binding of two MBL-associated serum proteases (MASP1 and -2), which leads to the subsequent activation of the complement cascade [61,62]. The main function of the relatively large collagen domain in SP-D and the closely related bovine proteins, CL-46 and conglutinin, is thought to be the proper spacing of the separate trimeric subunits in order to be able to cross-link carbohydrate structures present on the surface of separate microorganisms, leading to their subsequent aggregation and neutralization [63]. The positively charged collagen domain of membrane bound CL-P1 was suggested to be involved in the uptake of oxidized LDL particles [19].

After proper folding of the collagen helix, cysteine residues in the relatively short N-terminal domain (7–25 amino acids) form disulfide bridges between monomers, to stabilize trimeric subunits. The degree of multimerization differs between collectins, and it was demonstrated using chimeric collectin proteins, that the structural requirements for multimerization are located in the N-terminal cysteine-rich and in the collagen domain [63–66]. Deletion of particular cysteine residues within the N-terminal region leads to the formation of trimers only [38,44]. It is thought that in order to form multimers of the trimeric subunits, at least two cysteine residues have to be present in the N-terminal domain [38,44,53,67,68]. This view is supported by the fact that CL-L1, which has only one cysteine residue in this domain, is only present as a trimer [18]. However, CL-43 is secreted as a trimer only, despite having two N-terminal cysteine residues [69,70]. Moreover, the cysteine residues in CL-43 are found in exactly the same positions as in the highly multimerized SP-D [71]. Therefore, it is likely that in addition to the number of N-terminal cysteine residues, other factors also contribute to the oligomerization of trimeric subunits.

The collectins that form multimers of trimeric subunits can be divided into two groups. MBL and SP-A form octadecamers of six trimeric subunits, with their overall structure resembling a bouquet of flowers [51,72], whereas SP-D and the bovine proteins conglutinin and CL-46 are assembled into dodecamers of four trimeric subunits and form a cruciform-like structure [20,49,73] (Fig. 1). In addition, SP-D can form even higher-order multimers, so-called 'fuzzy balls' with a mass of several million kDa [73]. The size of fully assembled collectins ranges from 13 nm for MBL [51] to about 100 nm for SP-D [73]. These differences in size are determined mainly by the manner in which trimers are assembled into oligomers (bouquet of flowers vs. cruciform), and by the length of the collagen domains of the monomeric subunits. The exact sequences that determine these different arrangements of higher-order multimers remain to be identified.

Structural basis of monosaccharide recognition by collectins

Collectins require a broad monosaccharide specificity in order to recognize a variety of cell surfaces. This broad specificity is achieved by the fact that their CRDs have a very open trough-like binding pocket. This site selects its ligands mainly on the basis of the positioning of two vicinal hydroxyl groups, which form two coordination bonds with ligated calcium, four hydrogen bonds with calcium ligands and a single apolar Van der Waals contact [27,28]. Despite their broad monosaccharide specificity, C-type lectins, to which the collectins belong, can be divided into mannose/glucose-type or galactose-type, based on relative monosaccharide specificity. Specificity of the collectin CRDs for mannose over galactose is determined by three residues (Glu-Pro-Asn) at positions equivalent to the residues Glu185 and Asn187 in MBL [74–76]. Amino acid analysis and monosaccharide inhibition studies indicated that all collectins have mannose-type CRDs [75,77] with one exception, membrane-bound CL-P1, for which the amino acid analysis predicted preference of galactose over mannose [19]. Unfortunately, this predicted preference was not tested [19]. Although SP-A has a preference for mannose over galactose, its CRD contains the motif Glu-Pro-Arg, indicating that the conservation of the last amino acid of the triplet determining relative saccharide affinity is not critical [76]. However, substitution of the Glu-Pro-Asn (or Glu-Pro-Arg in the case of SP-A) triplet with Gln-Pro-Asp changes the CRD specificity from mannose-type to galactose-type [76], consistent with the fact that the latter triplet is conserved in the CRDs of galactose-recognizing C-type lectins [78,79]. At positions equivalent to the residues Glu185 and Asn187 in MBL, Glu and Ser are found in CL-L1 [18]. However, as extensive sugar binding studies are not yet available for this protein, the effect of the substitution of Asn by a Ser residue within the CRD on monosaccharide specificity is not known. Mutagenesis experiments have revealed that substitution of three amino acids and the insertion of a glycine-rich repeat, is sufficient to establish both high selectivity and affinity for galactose in CRDs normally recognizing mannose-type ligands [74,75,80]. Furthermore, the mode of galactose-binding was similar to the mode of ligand binding of the galactose-recognizing CRD from the asialoglycoprotein receptor [78].

The molecular basis on which CRDs discriminate between mannose- and galactose-type ligands lies in the presentation of two vicinal hydroxyl groups on the 3– and 4– position of the sugar ring of hexoses. For ligand binding in mannose-type CRDs, these hydroxyl groups need to have an equatorial position, whereas for high-affinity binding by galactose-type CRDs, they have to be placed axially. Interestingly, it is thought that fucose is bound by mannose-type CRDs in a slightly different manner, as this molecule has equatorial hydroxyl groups on its 2– and 3– positions of the sugar ring which, in molecular models, superimpose on the hydroxyl groups on the 3– and 4– position of the sugar ring of mannose [27,28,81]. In addition to fucose, αD-glucose also appears to be oriented differently from mannose within the mannose-type CRD. It was predicted recently, using computational docking studies, that αD-glucose docks into the SP-D CRD via vicinal equatorial hydroxyl groups on the 2– and 3– position of the sugar ring [82]. Although MBL has low affinity for the monosaccharide galactose, crystals of MBL complexed with this monosaccharide revealed that galactose was ligated in the MBL binding site via coordination bonds with equatorial hydroxyl

groups at the 1– and 2– position of the sugar ring [28]. This mode of binding excludes the possibility of binding to galactose residues in galactosides, as in this case the hydroxyl group at the 1– position of the sugar ring is involved in glycosidic bonding.

Binding of collectins to polysaccharides

Natural (poly)saccharide ligands for the collectins are normally attached to the surface of microorganisms, resulting in a high local density of collectin binding sites. High-affinity interactions between microorganisms and collectins depend on the density of carbohydrate ligands on the microbial surface [83] on the one hand, and on the degree of oligomerization of the collectin [66], on the other.

Clustering of glycoproteins or glycolipids on the surface of microorganisms allows for the simultaneous binding of multiple CRDs of one fully assembled collectin. In an elegant study by Lee *et al.* [83] using trimeric CRD/neck domains of MBL, it was shown that the affinity for monosaccharide subunits increased exponentially when these subunits were coupled to BSA, thereby increasing their surface density. The coupling of for instance 23 mannose monosaccharides per molecule BSA resulted in a decrease in the I_{50} value for this particular monosaccharide of about 85 000 times. In the same study it was found that the I_{50} values of various coupled monosaccharides differed dramatically: glucose was only slightly less potent than mannose in inhibiting MBL binding to a particular ligand when added as uncoupled monosaccharide, whereas when coupled to BSA, the inhibition potency differed by a factor 10. This clearly demonstrates the shortcomings of the use of monosaccharides in defining CRD specificity.

Biologically relevant interactions by collectins are brought about by the concerted binding to two or more monosaccharide units. It can be hypothesized that for native SP-A and MBL in their fully assembled form, in which the CRDs of multiple trimeric subunits all face the same direction, the affinity might be even further enhanced by simultaneously binding of up to 18 CRDs.

Multiple CRDs can also bind simultaneously to the monosaccharide units of a single polysaccharide chain. This follows from the observation that, when expressed per hexose unit, the mannose-polysaccharide mannan was more potent in inhibiting collectin binding to solid-phase bound ligands than mannose as monosaccharide. Part of this increased affinity may be explained by the interactions of (adjacent) saccharide units outside the CRD binding pocket. For SP-D it was shown by computational docking studies, that flanking saccharide residues in trisaccharides do form additional hydrogen bonds with amino acids outside the CRD binding pocket, and thereby contribute to overall binding energy [82]. The contributions of the flanking saccharides to overall binding energy was different for various trisaccharides, suggesting that amino acids outside the CRD binding pocket might be important in fine-tuning binding specificity of collectins, consistent with the fact that the amino acid residues at these positions are not conserved in collectins.

In addition to the surface density of carbohydrate ligands, the multimerization of collectins is of eminent importance for collectin binding to multivalent ligands. Compared with trimeric collectin subunits, monomers display rather weak affinity for immobilized saccharide ligands [33]: the K_d of the binding of a single C-type CRD with a monosaccharide ligand is in the order of 10^{-3} M, whereas the K_d of binding of collectin trimers and higher-order multimers to polyvalent ligands is in the order of 10^{-8} or 10^{-11} M, respectively [33,83].

Most studies concerning carbohydrate binding of collectins have focused on the binding to terminal carbohydrate residues. However, recently it was reported that the terminal sugar residues on lipopolysaccharide (LPS) of *Neisseria gonorrhoeae* and *Salmonella typhimurium*, could not always predict MBL binding [84]. Although direct evidence is lacking, this might suggest that MBL also interacts with internal sugar residues of LPS. In addition, SP-D has been shown recently to bind to nonterminal glucosyl residues of polysaccharides, and binding was shown to be dependent on the nature of the glycosidic linkage between monosaccharide units, as the hydroxyl groups on the 2– and 3– or on the 3– and 4– position had to be available to dock into the CRD [82]. Further studies are needed to see whether the ability to bind to internal saccharide units is a property of all collectins, or that it is specific for SP-D. Interactions of multiple CRDs of SP-D with one polysaccharide chain could be due to binding of two CRDs of one trimeric subunit and/or to binding of CRDs of different trimeric subunits. For instance, to bridge the 51 Å spanning region between CRDs within a trimeric subunit of SP-D, an oligosaccharide of 13 or 14 residues is needed, whereas bridging CRDs of different trimers would require a polysaccharide of up to 280 sugar residues to span the maximum distance of 100 nm between opposite sides of dodecameric SP-D. Binding of the collectins to multivalent ligands most likely requires some flexibility of the protein and/or the polysaccharide. Although it is not yet known whether the CRDs within trimeric subunits display substantial flexibility, electron microscopy pictures of dodecameric SP-D and conglutinin revealed great flexibility of trimeric subunits within these higher-order multimers [73]. For SP-A and MBL it is thought that the kink in the collagen stalk provides these oligomers with additional flexibility in order to bind to microbial surfaces [5,85]. In addition to flexibility on the part of the protein, NMR studies have shown that polysaccharide chains also have considerable flexibility [86,87] that might be of importance for collectin binding to these structures.

Functions of the collectins in host defense

Collectins interact with glycoconjugates and/or lipid moieties present on the surface of a great variety of microorganisms and allergens, and with receptors on host cells. Through these interactions, the collectins play an important role in innate host defense. The following host defense functions have been reported to date (Fig. 2).

Agglutination

Due to the formation of bridges between carbohydrate ligands present on the surface of different microorganisms, the interactions with intact microbes can result in massive aggregation [37,40,88–90]. This, in turn, may result in

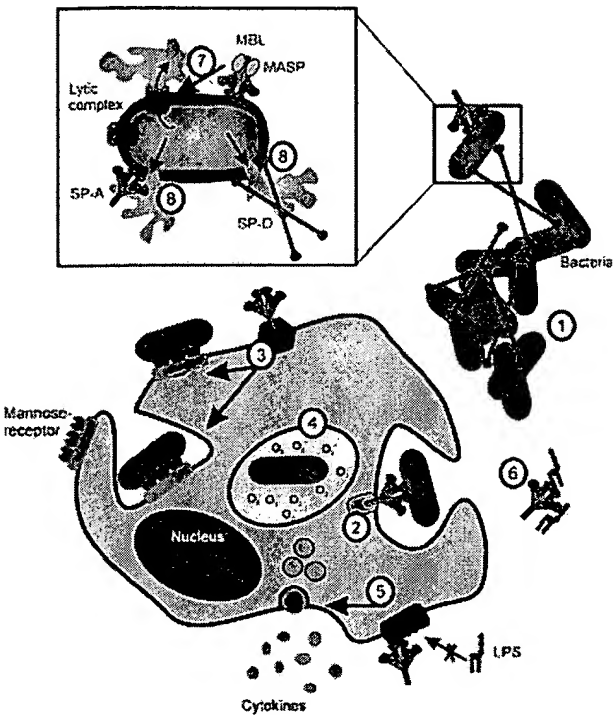

Fig. 2. Schematic representation of some of the functions of the collectins in innate immunity. For clarity, not all functions are shown for each collectin. Collectins aggregate microorganisms (1), and enhance phagocytosis of microorganisms by opsonization (2) or via indirect mechanisms, e.g. via upregulation of the activity of the mannose receptor (3). Collectins enhance the oxidative burst in phagocytes (4), and modulate the secretion of cytokines, e.g. via interaction with 'LPS-sensing' cell surface receptors (5), or by scavenging of LPS (6). MBL increases membrane permeability of microorganisms via activation of the lectin pathway of complement activation (7), while SP-A and SP-D increase membrane permeability via as yet unknown mechanisms (8). MASP, MBL-associated serine protease.

enhanced mucociliary removal by the respiratory tract, prevention of the attachment of pathogens to cell surfaces, and inhibition of microbial colonization and invasion. It may also facilitate uptake of the microorganisms by phagocytosis, but it should be noted that in some cases phagocytosis is decreased by agglutination [91,92].

Complement activation

Binding of MBL to microorganisms can result in inactivation of the organism by activation of the complement cascade [93,94]. On the other hand, by binding to C1q and thereby preventing association of C1q with C1r and C1s, SP-A can prevent the formation of active C1 complex [95].

Opsonization and activation of phagocytosis

Collectins may coat microorganisms and act as opsonins. This requires specific interactions of the collectins with receptors on phagocytic cells and may result in increased association, uptake and killing of the microorganisms [96–105]. Binding of MBL can lead to opsonization through complement activation and deposition of C3 [106], but can also opsonize microorganisms directly [107] as is the case for SP-A and SP-D. There is increasing evidence that, in addition to opsonization, where coating of microorganisms with collectins increases their uptake by phagocytes, SP-A and SP-D can also have direct, nonopsonic stimulatory effects on the uptake of microorganisms by phagocytic cells [40,97,108]. Binding to specific receptors on the surface of phagocytic cells may be responsible for this activation. At least one mechanism by which SP-A directly stimulates phagocytosis is by up-regulating the activity of the mannose-receptor, a pattern recognition receptor involved in the binding and phagocytosis of microorganisms [109].

Although in many cases SP-A stimulates phagocytosis and killing of pathogens, some microorganisms may increase the efficiency of their infection by using SP-A as a Trojan horse to gain entry to target cells [110–112]. MBL and conglutinin have been reported to enhance *in vivo* herpes simplex virus type 2 infection in mice [113].

Inhibition of microbial growth

Recent data indicate that collectins have direct effects on the survival of microorganisms. SP-A and SP-D were found to have direct effects on the survival of Gram-negative bacteria through mechanisms leading to increased permeability of the bacterial cell membrane [114]. Moreover, exposure of the facultative intracellular fungal pathogen *Histoplasma capsulatum* to SP-A or SP-D also resulted in increased cell permeability and enhanced killing of the pathogen [115], whereas SP-D has a pronounced inhibitory effect on the growth and hyphal outgrowth of the fungus *Candida albicans* [91].

Modulation of inflammatory responses

A considerable number of *in vitro* studies have focused on the modulation of inflammatory responses by collectins. Addition of MBL to blood from MBL-deficient donors decreases the secretion of tumor necrosis factor-α (TNF-α) by monocytes in response to *Neisseria meningitidis*, whereas MBL-induced alteration of interleukin (IL)-6 and IL-8 secretion was found to be concentration-dependent, with stimulation and inhibition by low and high concentrations of MBL, respectively [105]. MBL also inhibits release of TNF-α from human monocytes stimulated by rhamnose glucose polymers from streptococcal cell walls [116].

Also, SP-A and SP-D can modulate cytokine production [117–119]. These collectins can also modulate the production of reactive oxygen and nitrogen species, an important mechanism for killing of phagocytic cells [117–119]. In addition, SP-A and SP-D can act as chemoattractants for alveolar neutrophils or monocytes and thereby recruit the immune cells to the site of an inflammation [120–122].

Induction of inflammation by LPS or endotoxin, a component of the outer membrane of Gram-negative bacteria which is an important mediator of septic shock and acute respiratory distress syndrome, is dampened by SP-A or SP-D in a number of ways [123–126]. The mechanisms of this dampening include scavenging of the LPS [124] and binding to the LPS receptor CD14 on macrophages, which blocks LPS-mediated inflammatory responses of macrophages [126].

A matter of controversy in studies concerning SP-A has been whether this collectin should be considered anti-inflammatory or pro-inflammatory: some groups reported that interaction of SP-A with macrophages stimulates the production of proinflammatory mediators, such as TNF-α and NO, while others observed inhibition by SP-A of the production of these mediators (reviewed in [117–119]). A partial explanation of these conflicting results may come from recent observations that the functional outcome of SP-A exposure is determined by the state of macrophage activation. For example, SP-A enhances LPS-induced production of NO by interferon-γ (IFN-γ)-treated macrophages, while it inhibits LPS-induced NO production in macrophages not treated with IFN-γ [127]. In older experiments in which direct stimulatory effects of SP-A on cytokine release by macrophages was found, the result may have been due to contamination of the SP-A with LPS. However, Guillot *et al.* [128] showed that SP-A can stimulate cytokine secretion by macrophages, even when the SP-A has been treated with polymyxin to remove LPS. On the contrary, this was not seen by others using polymyxin-purified SP-A [129]. Differences in cell types and experimental variables may be the cause of this discrepancy.

A recent publication [130] provided evidence that SP-A and SP-D act in a dual manner to enhance or suppress inhibitory mediator production depending on binding orientation. The data in that paper indicate that SP-A and SP-D bind signal regulating protein α (SIRPα; a transmembrane protein involved in signal transduction) through their CRDs to initiate a signaling pathway that blocks proinflammatory mediator production. In contrast, their collagenous tails stimulate proinflammatory mediator production via binding to calreticulin/CD91. The authors [130] propose a model in which SP-A and SP-D help maintain a non/anti-inflammatory lung environment by stimulating SIRPα on resident cells via their CRDs. On the other hand, according to this model, interaction of these CRDs with pathogen-associated molecular patterns on foreign organisms or damaged cells and presentation of the collagenous tails in an aggregated state to careticulin/CD91 stimulates phagocytosis and proinflammatory responses.

In vivo studies using mice made deficient in SP-A or SP-D, show that the anti-inflammatory effects of both lung collectins predominate *in vivo*: exposure of SP-A –/– mice to intact microorganisms [131–133] as well as to LPS [125] results in increased inflammatory reactions in the lung compared to wild-type mice. Furthermore, increased pulmonary TNF-α concentrations, detected in SP-A –/– mice after exposure to LPS, could be normalized by the administration of exogenous SP-A [125]. *In vivo*, SP-D is thought to have an anti-inflammatory effect as well, because, compared to wild-type mice, SP-D –/– mice show increased inflammatory reactions in their lungs after infection with bacteria [133] or viruses [134].

Modulation of the adaptive immune system

In vitro, both SP-A and SP-D can inhibit the proliferation of T-lymphocytes, associated with a lowered IL-2 production [135,136]. Moreover, while SP-D enhances bacterial antigen presentation by bone marrow-derived dendritic cells [137], SP-A inhibits the differentiation of immature dendritic cells into mature dendritic cells [138]. *In vivo*, absence of SP-A in mice has effects on various lymphocyte subgroups [132].

Modulation of allergic response

The lung collectins SP-A and SP-D have been shown to mediate a number of anti-allergic effects [139–142], including inhibition of IgE binding to allergens, suppression of histamine release from basophils in the early phase of allergen provocation, and inhibition of lymphocyte proliferation in the late phase of bronchial inflammation.

Effects related to apoptosis

SP-A was reported to protect pulmonary alveolar type II epithelial cells from apoptosis [143]. In addition, there is evidence to suggest that MBL, SP-A and SP-D stimulate apoptotic cell clearance by alveolar macrophages [144,145].

Interactions with microorganisms and their carbohydrate surface epitopes

Numerous studies have demonstrated binding of collectins to the whole range of microbes, from viruses to metazoa. Microbial targets for SP-A have been listed in references [118,146–149]; those for SP-D in references [146,147, 149,150] and those for MBL, conglutinin, CL-43 and CL-P1 in [149]. Interestingly, in many cases, binding was found to be dependent on the growth conditions of the particular microbe, suggesting a complex interplay between host and microorganism. Most microorganisms display a diverse array of complex glycoconjugates on their outer surface, which represent possible ligands for the collectins. As most data are available for MBL and the surfactant proteins A and D, we will focus on these proteins.

Bacteria

Bacteria display on their outer surface an array of complex glycoconjugates, many of which are highly abundant or contain repeating saccharide units, thereby representing ligands for collectin binding. In several studies, the inability of collectins to bind to certain bacterial strains, correlated with increased pathogenicity [151]. In addition, capsule production by bacteria is often accompanied by decreased collectin binding and a subsequent increase in pathogenicity [98]. These findings clearly point to the importance of collectins in the early phase of host defense against bacteria.

In Gram-negative bacteria, LPS has been found to represent the most important ligand for collectin-mediated elimination. Initially it was thought that only bacteria displaying rough and not smooth LPS are bound by collectins. However, recently it was found that SP-A and SP-D bound both smooth and rough forms of *Pseudomonas aeruginosa*, suggesting that smooth LPS is recognized by both proteins on this type of bacteria [123]. In addition, SP-D does selectively bind to smooth forms of LPS expressed by O-serotypes of *Klebsiella pneumoniae* with mannose-rich repeating units in their O-polysaccharides [151]. In contrast, *K. pneumoniae* strains containing galactose-rich repeats in

their O-polysaccharides were not bound, in agreement with the known low affinity of SP-D for the monosaccharide galactose [151]. Rough forms of LPS act as a ligand for most collectins, although the latter bind to different sites on the LPS molecule: SP-A is thought to interact with the lipid-A moiety of LPS [152], whereas SP-D binds to LPS core saccharides [153]. On the other hand, it still needs to be elucidated which parts of the LPS molecule are involved in MBL binding. There are indications that besides the type of terminal sugar residue, also the folding of the LPS molecule is important [84]. Furthermore, it was found that the presence of glucose residues at the terminal LPS structure correlated with MBL binding, and more interestingly, a higher level of binding occurred to mutant forms of LPS terminating with heptose sugars [84]. However, monosaccharide inhibition studies using heptose sugars have not been performed so far, so it is still unclear whether these heptose sugars represent real MBL binding sites, or whether the observed correlation is coincidental. It is interesting to note that SP-A binds to *Haemophilus influenzae* not via its LPS, but instead via its glycosylated major outer membrane protein P2 [96].

There are also numerous Gram-positive bacteria that are bound by the collectins. The amount of data concerning ligands for the collectins on this type of bacteria is still very limited. However, we recently found that lipoteichoic acid (LTA) of *Bacillus subtilis* and peptidoglycan of *Staphylococcus aureus* represent ligands on Gram-positive bacteria for SP-D, but not for SP-A [154]. The structure of LTA varies among different strains of Gram-positive bacteria, whereas the structure of peptidoglycan in these bacteria is practically constant. Therefore, peptidoglycan may represent a universal ligand for SP-D. Although SP-A has been shown to bind to several Gram-positive bacteria [155], the surface structures that account for these interactions are as yet not known. In contrast, MBL has been shown to interact with a wide variety of Gram-positive bacteria [156,157], and various types of LTA were identified as MBL ligands [157].

The important lung pathogen *Mycobacterium tuberculosis* is bound by both SP-A and SP-D. To sustain a chronic infection and cause disease, *M. tuberculosis* needs to enter mononuclear phagocytic cells, where this pathogen survives by subverting cellular antimicrobial defense mechanisms [158]. While the interaction of *M. tuberculosis* with SP-D reduces the uptake of bacilli by macrophages [89], SP-A promotes this uptake [110]. Both proteins seem to interact with *M. tuberculosis* via lipoarabinomannan (LAM) molecules on their surface [89,159]. SP-A also binds to lipomannan (LM). Besides the presence of mannose residues on LAM and LM, fatty acids are an absolute requirement for SP-A binding [159]. SP-D interacted with *M. tuberculosis* via mannose residues of the LAM moiety of *M. tuberculosis* [41].

SP-D binds to *Mycoplasma pneumoniae* via interactions with its membrane glycolipids [160].

Viruses

Binding of collectins to viruses is especially interesting because viruses make use of the host cell machinery for the synthesis, folding and transport of proteins to the site of virus assembly at the cell surface. This machinery includes the array of biosynthetic and trimming enzymes responsible for attachment and processing of the oligosaccharides on their glycoproteins. No virus has been found to encode enzymes which can affect the glycosylation of its proteins by controlling commitment to particular processing pathways [161]. The dependence on host cell glycosylation machinery is demonstrated by the fact that infection of different cell types with, for instance, the respiratory viruses influenza A virus (IAV) or human respiratory syncytial virus (RSV) results in different oligosaccharide side-chains on their glycoproteins [162,163]. Most studies concerning the binding of collectins to IAV have used virus grown in embryonated hen eggs, which results in the expression of different oligosaccharide side-chains on the viral surface glycoproteins compared to IAVs grown in mammalian cells [163–165]. Moreover, most glycans of the hemagglutinin (HA)1 subunit have been identified as complex-type oligosaccharides, similar to that found on membrane-bound glycoproteins in mammalian systems [163]. It is therefore tempting to speculate that the acquisition of oligosaccharides antigenetically identical to those of the host helps the virus to escape the collectin-based immune defenses of the host organism, and is thus one of the mechanisms underlying antigenic drift [164].

Another interesting issue concerning the 'self' oligosaccharides exposed by many enveloped viruses, is how the collectins discriminate between 'self' oligosaccharides presented as part of the glycoproteins of the plasma membrane of the host cells and, the same oligosaccharides exposed on viral glycoproteins. One explanation might be that this discrimination is caused by a greater density of these epitopes in the latter situation. Furthermore, incomplete processing of the attached oligosaccharides, which increases the presence of oligosaccharides of the high-mannose type, might contribute to collectin binding to viruses. The presentation of oligosaccharides in a particular glycoprotein might further influence collectin binding. Although the carbohydrate structures present on viruses are of host origin, several lines of evidence suggest that collectins may play an important role in host defense against viral infections. These proteins bind to the enveloped viruses like IAV, herpes simplex virus type 1 (HSV-1), RSV, HIV, cytomegalovirus and the nonenveloped rotaviruses. Generally, collectins are thought to bind viruses or virus-infected cells in a manner that involves an interaction between the CRD of the collectin and surface-exposed glycoproteins containing oligosaccharides of the high-mannose type. In contrast, the binding of SP-A to IAV- and HSV-1-infected cells is mediated by interaction between the sugar binding activity of the virus and a carbohydrate moiety attached to SP-A [166,167]. The binding of SP-A to HSV-1 viral particles results in their enhanced uptake by alveolar macrophages [167]. Collectin binding to IAV has been extensively studied. MBL, SP-D, SP-A and conglutinin all display anti-IAV activity *in vitro*, although their method of action differs [88]. Although all collectins show inhibition of viral hemagglutination activity, SP-A was substantially less potent [88]. This lesser potency of SP-A might be caused by the different manner of interaction with the HA moiety of IAV. SP-D and conglutinin are thought to inhibit mainly viral replication by forming large viral aggregates. These

aggregates could then be removed via mucociliary clearance or by increased uptake by phagocytic cells. In addition, SP-D [168], MBL [169], conglutinin [170], but not SP-A [88], can prevent the IAV-induced inhibition of the superoxide production by neutrophils in response to the chemotactic peptide formylmethionylleucylphenylalanine, while SP-D [168], MBL [169] and conglutinin [170] enhance the IAV-induced H_2O_2 production by neutrophils. SP-D also increases the internalization of IAV by neutrophils [37, 168]. In contrast, MBL binding to IAV does not result in enhanced phagocytosis by neutrophils, but MBL dependent complement activation of IAV-infected cells [171] might contribute to the defense against IAV.

While SP-A binds to IAV through interaction between sialic acid residues on the carbohydrate moiety located in its CRD and (presumably) the sialic acid receptor present on the HA of IAV [166], SP-D from various species binds to IAV through interaction between the CRD of SP-D and oligosaccharide moieties located on the HA of IAV. Recently however, it was found that, like SP-A but in contrast to SP-D from all other animal species studied thus far, porcine SP-D contains a sialylated oligosaccharide moiety in its CRD [172,173]. This gives porcine SP-D an additional way of interacting with IAV: beside binding the carbohydrate moieties on HA of IAV, porcine SP-D can also bind IAV through interactions between the sialic acid residues on the carbohydrate moiety located in its CRD and the sialic acid receptor present on the HA of IAV. The presence of the sialylated oligosaccharide moiety enhances the anti-influenza activity of porcine SP-D, as demonstrated by assays of viral aggregation, inhibition of infectivity, and neutrophil response to IAV [174]. Hemagglutination inhibition assays revealed that porcine SP-D displays substantially greater inhibitory activity against various IAV strains than SP-D from other animal species [174]. The CRD carbohydrate of porcine SP-D is exclusively sialylated with α(2,6)-linked sialic acid residues [173]. Studies of the enzymatic modification of the sialic acid linkages present on porcine SP-D demonstrated that the type of linkage is important for hemagglutination inhibitory activity [173]. The more effective interaction between IAV and SP-D in the pig could result in a more effective clearance of IAV. Alternatively, however, it is conceivable that the more effective nonspecific immune response through SP-D in the pig could inhibit the induction of specific acquired immune responses which are elemental for the ultimate elimination of IAV. Evasion of IAV-induced immunity could thus give rise to conditions where IAV infection can persist. It is thought that pigs may act as 'mixing vessels' in which reassortment of IAV may occur upon coinfection with human and avian IAV strains [175]. The presence of the sialylated oligosaccharide in the CRD of porcine SP-D may therefore play a role in providing conditions by which pigs can act as 'mixing vessel' hosts that can lead to the production of reassortant, pandemic strains of IAV.

Ghildyal *et al.* [176] described that SP-A, but not SP-D and MBL, bound to respiratory syncytial virus (RSV). *In vivo*, SP-A was found to play an important role in the clearance of this virus [131]. In contrast to Ghildyal *et al.* [176], Hickling *et al.* [177] showed that SP-D did bind to RSV, and that the membrane envelope G-glycoprotein was involved in this interaction. Moreover, it was found in the same study that the trimeric recombinant head-neck fragments of SP-D had a protective effect on RSV infection *in vivo*, suggesting that multimerization of SP-D is not required for its protective role against RSV. It might be that carbohydrate moieties on the viral surface that are involved in receptor-mediated viral uptake by host cells, are bound by SP-D, thereby blocking viral entry into the host cell and subsequent infection [177]. Furthermore, it cannot be excluded that direct influences upon host cells are involved in the protective role of SP-D against viruses, e.g. by altering production of certain cytokines. The cause of the discrepancy between the data by Ghildyal *et al.* [176] and those of Hickling *et al.* [177] concerning SP-D binding to RSV is unclear.

MBL binds to HIV-1 and HIV-2 via gp120 and gp110, respectively. Both viral glycoproteins were found to contain oligosaccharide side-chains of the high-mannose type of 7, 8 or 9 mannose residues. The consequences of MBL binding to HIV are not known, but it could lead to neutralization of the virus via complement activation, or lead to enhanced uptake by phagocytic cells, and thereby, depending on whether the phagocytes are able to kill the virus after stimulated uptake, either enhance or diminish infection of the whole organism. Interestingly, the presence of sialic acid residues on the carbohydrate moiety of gp120 has been shown to decrease MBL binding, indicating that modification of the high-mannose oligosaccharides in the Golgi system may lead to modification of collectin-mediated defense against the virus [178,179].

Fungi

Most fungi are considered to be opportunistic pathogens, only causing disease in the absence of an adequate host immune response. An important site of entry for fungal infections is the lung. Therefore, most studies have focused on the effects and binding of the pulmonary collectins SP-A and SP-D to fungal pathogens. Possible binding sites for collectins on the surface of fungi can be divided into two groups. Firstly, structural polysaccharides consisting of repetitions of the same oligosaccharide elements can act as sites for collectin binding. In addition, many fungi express highly glycosylated proteins on their surface, which can also function as ligands for collectin binding. Some fungi produce a capsule, which is thought to represent a major virulence factor. Capsule production often leads to decreased collectin binding compared to acapsular fungal variants [90].

One of the first carbohydrate structures that was found to interact with the collectins was mannan, a structural component of the cell wall of the bakers yeast, *Saccharomyces cerevisiae*. Mannan is a branched homopolymer of mannose-residues that are coupled to each other via varying glycosidic linkages. SP-D binds and subsequently aggregates *S. cerevisiae* via binding with its C-type lectin domain [180]. Mannan and β(1–6)linked glucan represent major ligands for SP-D on the cell wall of *S. serevisiae*. Other structures involved could include mannoproteins. Interestingly, SP-A does not bind to *S. cerevisiae*, although SP-A does bind to its isolated cell wall component mannan [180]. The explanation for this apparent discrepancy may be that the specific mannan conformation on the yeast cell surface does not allow SP-A binding [180].

In addition to causing disease in immunocompromised individuals, *Aspergillus fumigatus* can cause allergen-induced allergic bronchopulmonary aspergillosis [181]. *A. fumigatus* conidia are bound by both SP-A and SP-D, and binding results in enhanced aggregation and killing by phagocytic cells [182]. Moreover, both pulmonary collectins interact with the glycosylated cell wall proteins gp55 and gp45 of *A. fumigatus*, inhibit specific IgE binding to these allergens and block histamine release from sensitized basophils [183]. The trimeric head-neck domain of SP-D was found to be enough to protect mice against fungal hypersensitivity [43,183]. Although the mechanisms are still unknown, these results clearly implicate pulmonary SP-A and SP-D in the modulation of allergic responses. In addition, after allergic airway inflammation caused by *A. fumigatus*, SP-D levels in bronchoalveolar lavage fluid were found to be increased [139,184].

SP-D binding to *Pneumocystis carinii* is mediated via interaction with the mannose-rich cell wall glycoprotein, gpA. Interestingly, pulmonary infection with this fungus leads to increased amounts of SP-D protein in bronchoalveolar lavage fluid [21], and the binding capacity of SP-D recovered from the bronchoalveolar lavage fluid of infected lungs is higher than that of recombinant SP-D, possibly due to its higher oligomerization state [36]. Although coating of *P. carinii* with SP-D was shown to increase the adhesion of fungal cells to macrophages [99], SP-D-induced aggregation seems to impair subsequent phagocytosis by alveolar macrophages [92]. The net effect of SP-D on *P. carinii* clearance *in vivo* is still unknown. SP-A-deficient mice show increased susceptibility to *P. carinii* infection [185], implying a role for SP-A in the host defense against this fungus *in vivo*. SP-A binds via its CRD to gpA on the surface of *P. carinii* [186], and in three studies, SP-A coating of *P. carinii* was shown to stimulate binding to alveolar macrophages, which supports the idea that SP-A functions as a nonimmune opsonin [99]. However, in another report, data indicated that SP-A decreased *P. carinii* attachment to alveolar macrophages and subsequent phagocytosis [187].

Binding of SP-D to *Candida albicans* not only induces aggregation of this organism, but more interestingly, coincubation of SP-D and *C. albicans* results in fungal growth inhibition, and decreased hyphal outgrowth, suggesting a direct effect of SP-D on fungal metabolism [91]. Furthermore, binding of SP-D to *C. albicans* inhibits phagocytosis of this fungus by alveolar macrophages [91], probably due to the large size of the formed *C. albicans* complexes, which are several times larger than alveolar macrophages. SP-A also binds to *C. albicans*, but phagocytosis of viable *C. albicans* by alveolar macrophages was not augmented [188]. In contrast, SP-A was found to inhibit increased phagocytosis induced by serum opsonization of *C. albicans* [188].

Both SP-A and SP-D bind to the yeast-like fungus *Cryptococcus neoformans*, although more binding was detected to the acapsular form [90,189,190]. In addition, mannoproteins of acapsular yeast cells and the major capsular component glucuronoxylomannan were identified as ligands for SP-D [190]. Binding of SP-D to *C. neoformans* leads to a massive aggregation of acapsular but not of encapsulated *C. neoformans*. Moreover, secreted glucoronoxylomannan can inhibit the SP-D induced aggregation [190]. Binding of SP-A to *C. neoformans* does not result in the increased uptake by phagocytic cells [189], in analogy with the effect of SP-A binding to *C. albicans* [188]. MBL was found to bind to *C. albicans* and acapsular *C. neoformans* [191]. Unfortunately, possible effects of these interactions were not studied.

Parasites

MBL binds to a number of blood stage protozoa, including *Plasmodium falciparum*, *Trypanosoma cruzi*, and several *Leishmania* species. Glycolipids and N-linked glycans of the high-mannose type were identified as potential ligands on their surface [192–194]. *Leishmania* species are intracellular pathogens, mainly infecting macrophages. Several lines of evidence indicate that this parasite uses the lectin pathway of complement activation to its advantage. To enter macrophages, it uses the coating of its surface with complement, which stimulates its uptake via complement receptors on the surface of macrophages [195,196]. Therefore, MBL potentially provides a mechanism for cell entry via activation of the lectin-pathway of complement activation. This hypothesis is supported by the observation that there is a correlation between the plasma MBL concentration and the susceptibility to visceral leishmaniasis [197]. Interestingly, intracellular *Leishmania mexicana* amastigotes secrete a structure called proteophosphoglycan, which is bound by MBL, resulting in turn in the activation of the complement cascade [198]. As activation of the complement cascade results in the release of several pro-inflammatory peptides, it is thought that this is a mechanism used by the parasite to attract infectable monocytes to the site of infection [198]. MBL also binds to several developmental stages of the multicellular blood fluke, *Schistosoma mansoni*, and at least *in vitro*, this binding results in the activation of the complement cascade [199]. In addition, we recently demonstrated SP-D binding to specific larval stages of *S. mansoni* that are known to migrate through the lung [200].

Interactions of collectins with host cells

Collectins also display specific interactions with host cells. Immune cells are the most frequently studied cells in this respect, although for SP–A interactions with type II alveolar cells have also been studied in great detail. An important function of collectins is their ability to enhance phagocytosis of microorganisms. The mechanisms by which they stimulate the uptake of specific pathogens include opsonization of microorganisms [96–105], as well as direct interactions with phagocytic cells [40,97,108]. Stimulation of phagocytosis through opsonization by collectins is in most cases mediated via their CRD-dependent binding to microorganisms, after which specific cellular receptors are involved in the internalization of the collectin-coated microorganisms. Although an increasing number of receptors for collectins have been identified on host immune cells over the last decade (Table 2), the picture is far from complete.

Because of the structural similarity between C1q and the collectins MBL and SP-A, one of the first receptor types identified as a general collectin receptor involved in the collectin-mediated stimulation of phagocytosis was the C1q receptor, later identified as calreticulin [212]. It was found

Table 2. Binding of collectins to cell surface receptors and the biological consequences. Yes, direct binding detected; No, direct binding studied, but not (yet) detected; ?, no information available in literature about direct binding. LPS, lipopolysaccharide; TNF-α, tumor necrosis factor α; cC1qR, C1q recepor, also known as cell surface calreticulin; C1qRp, C1q receptor stimulating phagocytosis; CR1, complement receptor 1; SIRPα, signal regulating protein α; SP-R210, surfactant protein receptor of 210 kDa; gp-340, glycoprotein 340; TLR2, toll-like receptor 2; TLR4, toll-like receptor 4.

	MBL		SP-A		SP-D	
Receptor	Binding	Mediates	Binding	Mediates	Binding	Mediates
cC1qR (calreticulin)	Yes [57]	Phagocytosis of apoptotic cells [201]	Yes [57]	Phagocytosis of microorganisms [58] Phagocytosis of apoptotic cells [144]	No [57]	Phagocytosis of apoptotic cells [144]
C1qRp	?	Phagocytosis of microorganisms [202]	?	Phagocytosis of microorganisms [202]	?	?
CR1	Yes [203]	Phagocytosis of microorganisms [203]	?	?	?	?
CD14	Yes [204]	?	Yes [126, 205]	Modulation of LPS-elicited cytokine release [205]	Yes [126]	Inhibition of LPS-elicited cytokine release [126]
SIRPα	?	?	Yes [130]	Inhibition of LPS-elicited cytokine release [130]	Yes [130]	Inhibition of LPS-elicited cytokine release [130]
SP-R210	?	?	Yes [206]	Phagocytosis of microorganisms [207] Inhibition phospholipid secretion by alveolar type-II cells [206] Enhancement of nitric oxide production [208] Enhancement of TNF-α production [208] Inhibition of T-lymphocyte proliferation [135]	?	?
gp-340	?	?	Yes [209]	?	Yes [210]	?
TLR2	?	?	Yes [211]	Inhibition of peptidoglycan-elicited cytokine release [211]	?	?
TLR4	?	?	?	Stimulation of cytokine synthesis [128]	?	?

that MBL, SP-A and conglutinin interact with this receptor, while binding could be inhibited using C1q and C1q collagen stalks, demonstrating that the collectin collagen domain is involved in receptor binding [56,213]. SP-A-induced phagocytosis of *S. aureus* by monocytes was shown to be dependent on the presence of the C1q receptor on the cell surface [58]. In addition, SP-A-mediated attachment of *M. tuberculosis* to alveolar macrophages was shown to be inhibited by type V collagen, suggesting that the C1q receptor was involved [214,215]. SP-D can bind in a lectin-independent manner to alveolar macrophages [213], but in contrast to the binding of other members of the collectin family, the C1q receptor appears not to be involved in this interaction [216,217]. More recently, it was demonstrated that collectins stimulate the phagocytosis of apoptotic neutrophils [145] and jurkat cells [145,201] by (alveolar) macrophages. Collectins are thought to bind to apoptotic cells via CRD dependent mechanisms, whereas they probably interact with their collagen domain to the cell surface calreticulin/CD91 complex on macrophages, after which ingestion starts [201]. As calreticulin lacks a transmembrane domain the endocytic receptor protein CD91 is thought to be involved in the transduction of the signals initiating engulfment after the calreticulin/collectin complex has been formed [144,201]. *In vitro*, SP-A was found to be more potent than SP-D in stimulating engulfment of apoptotic cells by alveolar macrophages [145], whereas using knockout (*SP-A* or *SP-D*) and overexpressing (*SP-D*) mice, only SP-D was found to alter apoptotic cell clearance from naive murine lung, suggesting that SP-D plays a particularly important role *in vivo* [144]. In support of the suggested role of SP-D in the clearance of apoptotic cells, is the finding that in SP-D –/– mice an increased number of apoptotic alveolar macrophages is present, whose number is reduced by the intrapulmonary administration of a head and neck fragment of SP-D produced by recombinant techniques [218].

The structure of an additional C1q receptor was more recently elucidated, and demonstrated to be a highly glycosylated protein of 126 kDa. Due to its demonstrated involvement in the enhancement of phagocytosis of C1q- and collectin-opsonized microorganisms, it was named C1qRp (C1q receptor stimulating phagocytosis). Although direct binding of the collectins to this receptor has never been demonstrated directly, sequestration of the receptors using specific antibodies directed to this receptor, decreased collectin-mediated phagocytosis [202]. C1qRp is expressed in cells of myeloid origin, platelets and on endothelial cells [219]. It should be noted that Tino and

Wright [220] demonstrated that stimulation of phagocytosis of specific pathogens by SP-A is inhibited in monocytes adhering to surface-bound C1q, but not to similarly treated alveolar macrophages. This suggests that depending on the cell type, receptors other than C1qR and C1qRp are also involved in collectin-based stimulation of phagocytosis.

MBL has been shown to interact with the complement receptor 1 (CR1/CD35) in a CRD-independent manner that was inhibitable by C1q [203]. Furthermore, it was shown that CR1 was involved in the MBL-enhanced phagocytosis by neutrophils of *Salmonella montevideo* suboptimally opsonized with IgG [203].

MBL, SP-A and SP-D have all been shown to bind directly to the cell surface LPS receptor CD14. However, the specific domains, on both the receptor and the collectin, involved in these interactions differ for the various collectins: MBL and SP-A bind to the peptide portion of CD14, whereas SP-D binds to the N-linked glycan moiety of CD14 [126,204]. Moreover, the neck domain of SP-A was shown to be involved in SP-A binding to CD14 [126], whereas for SP-D its CRD mediated binding to this receptor [126]. The domain of MBL involved in CD14 binding has not yet been identified, but is probably not its CRD, as binding of MBL to CD14 could not be inhibited by competing sugars and the presence of EDTA [221]. The modulation of cellular effects elicited by the above mentioned collectins also varies upon stimulation with different bacterial membrane products. The fact that the type of LPS influences SP-A binding and subsequent receptor stimulation was clearly demonstrated by Sano *et al.* [205], who showed for alveolar macrophages, that SP-A inhibited TNF-α secretion elicited by smooth LPS, probably via binding of SP-A to CD14, thereby preventing smooth LPS from interaction with this receptor. However, SP-A binding to rough LPS enhanced the interaction of the LPS with CD14 and subsequent TNF-α release. In contrast to this study, Stamme *et al.* [222] found that SP-A could prevent the rough LPS-induced translocation of the transcription factor NF-κB, which is known to stimulate the secretion of TNF-α. This inhibitory effect was most probably caused by preventing the formation of LPS/LPS-binding protein complexes. The cause of this contrast in observations is not exactly known, but may include the use of different types of rough LPS and differences in the percentage of serum (containing LPS binding protein) that were used in both studies. For SP-D the picture was less complex, as this protein was shown to inhibit the binding of both smooth and rough LPS to CD14 [126]. Although MBL has been shown to bind to CD14 as well as to streptococcal rhamnose/glucose polymers, this protein inhibited the interaction of these bacterial membrane products with CD14 on human monocytes, thereby preventing the subsequent release of TNF-α [116].

It was reported recently [130] that SP-A and SP-D inhibit LPS-induced macrophage cytokine release by interacting via their CRD with signal regulating protein α (SIRPα). This is a transmembrane protein involved in signal transduction that contains a glycosylated extracellular region [223]. How the effect of SP-A via SIRPα is related to the observed effects brought about via binding of SP-A to CD14 and LPS [205,222] remains to be determined.

Besides the function of SP-A in innate immunity, *in vitro* studies have demonstrated that this protein can inhibit phospholipid secretion by lung alveolar type II cells, and that a specific SP-A receptor with a molecular mass of 210 kDa, designated SP-R210 is involved in this inhibition. Although binding of SP-A to SP-R210 was shown to require the presence of calcium, binding could not be inhibited by mannan, suggesting that the lectin activity of SP-A is not responsible for the observed interaction [206]. In addition to its presence on alveolar type II cells, SP-R210 has been detected on macrophages and the macrophage cell-line U937 [206]. Moreover, for alveolar macrophages it was shown that phagocytosis and subsequent killing of SP-A-opsonized *M. tuberculosis* was dependent on the presence of this receptor on their cell surface [208]. SP-R210 is also involved in inhibition of T cell proliferation by SP-A, via an interaction with the SP-A collagen domain, most probably involving a highly charged RGD motif [135].

On bovine alveolar macrophages, an additional SP-A-specific receptor was shown to be present. SP-A bound to this 40-kDa protein in a calcium-dependent and mannose-inhibitable manner, indicating that the CRD of SP-A is involved in interactions with this receptor [224]. Unfortunately until now, no data are available about possible functions of this 40 kDa SP-A receptor.

Glycoprotein-340 (gp-340) has been shown to interact with both SP-A and SP-D. Although this interaction was calcium-dependent, it did not involve the lectin activity of SP-A or SP-D [209,210], suggesting a protein–protein interaction. Furthermore, the expression of gp-340 and SP-D colocalize throughout the body, suggesting that SP-D has a role as an opsonin receptor [225].

As suggested by Kuan *et al.* [226], the fact that SP-D binding to macrophages is, for a large part, mediated via its CRD might indicate that besides the above mentioned receptors, glycolipids are also involved in SP-D binding to these cells.

SP-A has recently been shown to bind via its CRD to toll-like receptor (TLR)2, thereby preventing the induction by peptidoglycan (a cell wall component of Gram-positive bacteria) of TNF-α secretion by U937 and alveolar macrophages [211]. It was demonstrated recently that SP-A-induced cytokine synthesis in mouse macrophages is critically dependent on functional TLR4 [128], but it remains to be determined whether a direct interaction between SP-A and TLR4 is involved in this effect.

SP-A and SP-D have been shown to stimulate chemotaxis of alveolar macrophages [122,227], but not of peripheral blood monocytes [227], suggesting the presence of specific cellular receptors on the former cell type. The effect of fully assembled SP-A and SP-D on chemotaxis correlated with their ability to stimulate directional actin polymerization [227]. Interestingly, the ability of both SP-A and SP-D to stimulate chemotaxis of alveolar macrophages did not seem to be mediated by the lectin activity of both proteins, as it was not inhibitable by sugars, and for SP-A at least partly mediated via its collagen domain [122]. Cai and coworkers [42] demonstrated that the trimeric head-neck domain of SP-D was sufficient to stimulate chemotaxis of peripheral neutrophils, and that this effect could be blocked by maltose, strongly suggesting that SP-D-induced chemotaxis of this cell type is stimulated by CRD-dependent

interactions. These conflicting results concerning the involvement of CRD in the mediation of chemotaxis might be explained by the assumption that the chemotactic effect in both cell types is exerted by different cellular receptors. Although SP-A does not directly stimulate neutrophil chemotaxis, this protein does alter neutrophil responsiveness to chemoattractants: SP-A was found to enhance chemotaxis of inflammatory alveolar neutrophils, whereas it has the opposite effect on peripheral neutrophils [228]. In agreement with a function of SP-A in chemotaxis within the lung is a recent report demonstrating that SP-A stimulates the recruitment of neutrophils in the lungs of preterm lambs [229]. In SP-A –/– mice, there is an increased influx of immune cells into the lung upon infection, suggesting that SP-A decreases the influx of these cells into the lung. However, this increased influx of immune cells might be caused by secondary changes in the SP-A –/– mice, e.g. altered cytokine levels. In contrast to the chemotactic effect of both lung collectins, MBL does not seem to directly stimulate chemotaxis [227].

Conclusions and future directions

Collectins play an important role in innate immunity. Although most functional information regarding collectins is based on *in vitro* experiments, more recently, knock-out mice have become available for SP-A [230], SP-D [231,232] and MBL-A [233] that support the generally accepted concept that the main function of these proteins lies in the innate immunity against microorganisms [131,133,134, 185,233]. In addition, the fact that in humans mutations within the *MBL*-gene influence MBL serum levels, and at the same time susceptibility to certain pathogens (as reviewed by Turner *et al.* [234]), supplies additional evidence that this molecule is important in innate immunity. Collectins exert their role via a diversity of mechanisms, and they interact with surface structures present on host cells as well as on microorganisms. Binding of collectins to microorganisms often occurs via CRD-dependent interactions with glycoconjugates on their surface. On the other hand, binding to host cells is more complex, including both CRD-dependent interactions with surface glycoconjugates as well as protein–protein interactions that involve, for instance, the collectin collagen or neck domain.

Monosaccharide inhibition studies have revealed that collectins preferentially bind to monosaccharide units of the mannose-type. At present a great amount of data is available at the molecular level regarding the manner in which collectins bind their target monosaccharides. This not only increases our understanding why some pathogens are bound by the collectins while others are not, but may in the future allow for the development of recombinant proteins which have altered carbohydrate specificity. It is known, for instance, that a large part of the cases of pneumonia are caused by *K. pneumoniae* serotypes containing galactose-rich O-antigens on their LPS [151]. These bacteria have been found to be relatively resistant to SP-D-mediated inactivation *in vitro* [151]. Therefore, recombinantly produced SP-D, in which the carbohydrate specificity is altered from mannose-type to galactose-type, might have a therapeutic value for patients suffering from pneumonia caused by these types of bacteria. This hypothesis might be broadened to other collectins. As a first step to evaluate this hypothesis, it could be considered if recombinant collectins with galactose specificity can inactivate these high-galactose containing serotypes of *K. pneumoniae* by aggregation or stimulation of their phagocytotic uptake. Concerning the inactivation of microorganisms normally resistant to collectin mediated inactivation, additional experiments in which, for instance, SP-D with altered carbohydrate specificity is expressed in the SP-D knockout background could elucidate the effects of galactose-specific collectins *in vivo*. Furthermore, expressing SP-D with galactose specificity in this SP-D –/– background could provide information on whether the mannose specificity is required to restore normal surfactant homeostasis, and prevent the development of lung emphysema, alterations normally seen in the SP-D –/– phenotype. Many glycoconjugates present on the surface of host cells contain galactose units. Therefore the effects of collectins with altered carbohydrate specificity on these cells need to be investigated as well.

Although the list of microorganisms and their respective carbohydrate surface ligands that are bound by the collectins steadily increases, the exact pathways that result in the distinct effects of the different collectins upon binding to various microorganisms, are as yet not clear. It is for instance intriguing that binding of SP-D to *K. pneumoniae* results in their increased phagocytosis by alveolar macrophages [98], while binding of SP-D to *M. tuberculosis* has the opposite effect [41,89]. Therefore, a challenging task for the future will be to elucidate the mechanisms by which collectin binding to one type of pathogen leads to its removal, while binding to another increases its infectivity.

Recently, a number of receptors have been identified on the surface of host cells. However, as yet, not all collectin-mediated effects upon host cells can be explained by interactions with these receptors. A large amount of work still needs to occur in order to link collectin-mediated effects upon host cells to already identified receptors, as well as to identify additional collectin receptors. A better understanding of collectin-mediated immunity may in the future allow the identification of disease states in which the therapeutic administration of collectins may be beneficial.

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EXHIBIT B

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Review

Collectins and ficolins: sugar pattern recognition molecules of the mammalian innate immune system

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Abstract

Collectins and ficolins represent two important groups of pattern recognition molecules, which bind to oligosaccharide structures on the surface of microorganisms, leading to the killing of bound microbes through complement activation and phagocytosis. Collectins and ficolins bear no significant sequence homology except for the presence of collagen-like sequences over the N-terminal halves of the polypeptides that enable the assembly of these molecules into oligomeric structures. Collectins and ficolins both contain lectin activities within the C-terminal halves of their polypeptides, the C-type carbohydrate recognition domain (CRDs) and fibrinogen β/γ (homology) (FBG) domain, respectively. These domains form trimeric clusters at the ends of the collagen triple helices emanating from a central hub, where the N-terminal ends of the polypeptides merge. The collectins and ficolins seem to have evolved to recognize the surface sugar codes of microbes and their binding, to these arrays of cell surface carbohydrate molecules, targets the microbe for subsequent clearance by phagocytic cells.

Keywords: Collectin; Ficolin; Lectin; Innate immunity; Complement; Pattern recognition

1. Introduction

Clonal immunity is equipped with lymphocyte antigen receptors that are able to recognize diverse and highly specific antigens. However, these receptors only effectively detect antigens after the raw antigens, for example, microbes, are processed and presented on antigen-presenting cells (APCs) as short antigenic epitopes. The APCs, for example, macrophages and dendritic cells, do not possess the antigen receptors that are expressed by lymphocytes. Instead, these phagocytes directly or indirectly employ a limited number of germline-encoded receptors or soluble molecules for pathogen recognition [1]. These molecules appear to have evolved to distinguish microbial pathogens from self-antigens through the recognition of molecular arrays, called pathogen-associated molecular patterns (PAMPs), which are essential to the survival of certain microbial groups, for example, Gram-positive vs. Gram-negative, and are, therefore, highly conserved among the different classes of pathogens. These pathogen recognition molecules are called 'pattern recognition receptors' (PRRs) [1]. PAMPs can be protein, lipid, nucleic acid, and carbohydrate. The Toll-like receptors (TLRs) are a large family of PRRs that recognize PAMPs of diverse chemical nature. An increasing number of mammalian lectins form another class of PRRs that bind specifically to the unique carbohydrate moieties on microbes [1–7].

The collectins belong to the Ca^{2+}-dependent (C-type) lectin superfamily characterized by the presence of the C-type carbohydrate recognition domain (CRD) [2,6]. The ficolins possess a different type of lectin domain, called the fibrinogen-like [fibrinogen β/γ (homology) or FBG] domain [8,9]. Collectins bind to a wide range of sugar residues in a Ca^{2+}-dependent manner. Ficolins also bind to sugar residues that are rich on microbial surface, for example, *N*-acetyl-D-glucosamine (GlcNAc) in a calcium-independent manner [10–13]. The collectins and ficolins

Abbreviations: CRD, carbohydrate recognition domain; FBG, fibrinogen β/γ (homology); PAMP, pathogen-associated molecular patterns; LPS, lipopolysaccharide; LTA, lipoteichoic acid; PRR, pattern recognition receptor; TLR, Toll-like receptor; MBL, mannan-binding lectin; SP-A, surfactant protein A; SP-D, surfactant protein D; MASP, MBL-associated serine protease

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bear no significant similarity in amino acid sequences. However, the two groups of proteins have similar domain organizations and assemble into similar oligomeric structures. Functionally, both collectins and ficolins mediate microbial killing through similar effector systems. This review will discuss the basic structural and functional properties of these two groups of lectins. A number of reviews have been published on various aspects of collectins and ficolins [3–8,14,15]. Three other articles in this issue are dedicated respectively to overviews on animal lectins, the C-type CRD and a specific account of the clinical aspects of the collectin mannan-binding lectin (MBL) [16–18].

2. General features

The collectin family has five well-characterized members, that is, MBL, lung surfactant protein A (SP-A) and D (SP-D), bovine conglutinin and collectin-43 (CL-43) [3–6]. Recently, another novel collectin, called collectin liver 1 or CL-L1, has been cloned which is expressed in most tissues except skeletal muscle [19]. The ficolin family consists of three members, that is, H-ficolin initially known as Hakata autoantigen [13], L-ficolin also known as p35 [10], and M-ficolin [12]. The tissues of origin and presentation and carbohydrate binding properties of these molecules are summarized in Table 1. It should be noted that bovine conglutinin and CL-43 are only detected in the bovidae [20,21].

MBL is synthesized in the liver and secreted to the blood circulation. The serum concentrations of MBL are highly variable in healthy populations [22,23] and this is genetically controlled by the polymorphism/mutations in both the promoter and coding regions of the MBL gene [24–26]. As an acute phase protein, the expression of MBL is further upregulated during inflammation [27]. Both SP-A and SP-D are mostly synthesized in the lungs by type II alveolar cells and Clara cells and are secreted into the alveolar space. SP-A was initially identified in the lung surfactant as a relatively hydrophilic protein component, which also appears in amniotic fluid [28,29]. Recently, SP-A or SP-A-like proteins have been detected at mRNA and/or protein levels in extrapulmonary tissues such as prostate, thymus, intestinal mucosae, the Eustachian tube, middle ear, paranasal sinuses, mesothelium and synovium [6,30–32]. SP-D was initially identified from primary culture medium of type II alveolar cells [33]. SP-D association with the lung surfactant is probably mediated by SP-D binding to the surfactant lipid, phosphatidylinositol, via its CRD and it can be eluted from the surfactant with EDTA [34,35]. In fact, a large fraction of

Table 1
Expression and sugar specificity of collectins and ficolins

	Other names	Tissues of origin	Tissues of presentation	Sugar specificity
Collectins				
MBL	MBP, RaRF(p28)	liver (hepatocytes)	serum	GlcNAc>mannose/L-fucose> ManNAc≫maltose>glucose
SP-A	SAP-35, SP28–35	lung epithelium, prostate, thymus, intestinal mucosa, the Eustachian tube, middle ear, paranasal sinuses, mesothelium, synovium	bronchus, alveolus, mucosal surfaces, semen	ManNAc>L-fucose/maltose> glucose>mannose/galactose (?)
SP-D	CP4	lung epithelium, gastrointestinal epithelium, and other tissues [33]	bronchus	Maltose>L-fucose>mannose> glucose≫glucosamine
conglutinin		bovine liver	serum	GlcNAc≫mannosamine> L-fucose/mannose>glucose> ManNAc/glucosamine>Maltose
CL-43		bovine liver	serum	Mannose/manNAc>L-fucose> GlcNAc>glucose/maltose> galactose>lactose≫GalNAc
CL-L1		tissues except skeletal muscle	n.d.	mannose
CL-P1		vascular endothelial cells, placenta	endothelial cells	n.d.
Ficolins				
H-ficolin	Hakata antigen	liver (hepatocytes and bile epithelium), type II alveolar cells, ciliated bronchial epithelial cells	serum, bronchus, alveolus, bile	GlcNAc, GalNAc, glucose
L-ficolin	p35, huculin, EBP-35	liver	serum	GlcNAc
M-ficolin	ficolin-1, ficolin-α, ficolin-β	uterus, monocytes	uterus membrane, monocyte surface	GlcNAc

GlcNAc, *N*-acetyl-D-glucosamine; GalNAc, *N*-acetyl-D-galactosamine; ManNAc, *N*-acetyl-D-mannosamine. n.d., not determined. (?) indicates controversial reports on binding of SP-A to galactose. Like the collectins, CL-P1 contains collagen-like sequences and a C-type CRD [134]. However, it is distinct from collectins in that it is membrane-anchored and it forms a triple coiled-coil at the end of the collagen triple helix that is opposite the CRD. In collectins, a relatively shorter triple coiled-coil forms between the collagen helix and CRD. Compared with the permanent oligomers seen in collectins, CL-P1 can potentially form transient clusters at the pathogen–host cell interfaces like the mannose receptor.

soluble SP-D is found in the lung lavage. In contrast, SP-A is more tightly associated with the surfactant lipids and can only be effectively separated from the surfactant lipids by organic solvent extraction or chaotropic treatment. Like SP-A, SP-D is also expressed in many extrapulmonary tissues including the gastrointestinal mucosa [6,36]. Bovine conglutinin was the first member of the collectin family to be characterized, being initially identified as a bovine serum component that could agglutinate horse serum-reacted guinea pig erythrocytes [37]. It is probably also the first animal protein that was demonstrated to have lectin activity when Leon and Yokohari [38] showed that binding of conglutinin to complement-reacted erythrocytes was inhibitable with acetamido sugars such as GlcNAc. Conglutinin is synthesized in the liver but it is only found in the bovidae. CL-43 is also found exclusively in the bovidae as a serum protein synthesized in the liver [39].

Ficolins were first purified from porcine uterus as membrane-associated proteins that bound to transforming growth factor β1 (TGF-β1) [9]. The lectin activity of the ficolins was first described for L-ficolin, or p35, based on its affinity for the yeast cell wall carbohydrate structure, mannan [10]. H-ficolin was initially identified as the Hakata autoantigen, recognized by autoantibodies in the sera of systemic lupus erythematosus patients, and its lectin activity was consistent with its structural similarity to L- and M-ficolin [13]. H-ficolin is synthesized in the liver by hepatocytes and the bile duct epithelial cells and is secreted into both the blood circulation and bile [40]. It is also synthesized in the lung by ciliated bronchial epithelial and Type II alveolar cells and secreted into bronchus and alveolus [40]. M-ficolin is so named for its predominant expression in circulating blood monocytes [41]. The tissue presentation of M-ficolin remains to be defined. However, it is detectable on the surface of monocytes despite the lack of typical membrane anchoring signals [42].

3. Structure

3.1. Primary structural features

The structures of collectins and ficolins are primarily defined by the unique domain organization of the polypeptide sequences. A collectin polypeptide consists of four distinct regions. This includes a short N-terminal segment of 7–28 amino acid residues in which 1–3 cysteine residues are found, followed by a 53–177 residues collagen-like region characterized by the $(-Gly-Xaa-Yaa-)_n$ repeating sequence (Fig. 1). MBL and SP-A are much shorter than SP-D, conglutinin and CL-43 over this region. The collectin polypeptides end at the C-terminus with C-type CRDs that are connected to the collagen-like region through a short 'neck' region of 24–28 residues. The collagen-like regions form triple helices and the 'neck' regions form stable, trimeric, α-helical coiled-coils [43].

The ficolin polypeptides have similar domain organizations except the lack of the 'neck' sequence. At their C-terminal ends, ficolins have the FBG domain. Ficolins possess an N-terminal segment of 23–28 residues and 2 cysteine residues are found in this segment. Ficolins typically have shorter collagen-like sequences (11–17 Gly–Xaa–Yaa repeats) than collectins ranging from 33–51 residues or 11–17 Gly–Xaa–Yaa repeats. MBL has the shortest collagen-like region in collectins, which is 60 residues or 20 Gly–Xaa–Yaa repeats. This difference in the length of the collagen-like regions of collectins and ficolins are reflected in the different dimensions of the molecules (Fig. 1).

3.2. Tertiary structure

The tertiary structure of collectins and ficolins resembles to that of complement protein, Clq in overall structure [44,45]. The oligomeric nature of these molecules was mostly evident from the mobility of the proteins on SDS-PAGE under reducing and non-reducing conditions. All collectins and ficolins migrate as high molecular weight bands under non-reducing conditions and are, upon interruption of disulfide linkages, reduced to single polypeptide species of 30–45 kDa [10,13,21,46–48]. The degree of inter-chain disulfide bonding, for example, complete or partial, can be estimated by comparing the molecular weights of the molecules, under non-reducing conditions, with that of a single polypeptide.

Electron microscopy has been used to determine the overall structure of collectin and ficolin molecules. Conglutinin was the first collectin found to contain collagen-like sequences and to assume a highly oligomerized structure as viewed under the electron microscope [46,49]. Conglutinin, when examined by SDS-PAGE, is a disulfide-linked oligomer, larger than 300 kDa under non-reducing conditions and it migrates as a 45-kDa polypeptide under reducing conditions [46,47]. A 43-kDa peptide is often observed which is now known to be a degradation product of the 45-kDa polypeptide due to a single cleavage in the collagen-like region [47]. These clipped conglutinin molecules are observed as monomers showing a large globular region at one end, corresponding to a cluster of three CRDs, whereas an intact conglutinin molecule consists of four such structures with the smaller ends merging into a central knob and the large clusters of CRDs radiating toward the periphery [47]. Under non-reducing conditions, in SDS-PAGE, the degraded conglutinin molecules were observed as a 43-kDa polypeptide showing that inter-chain disulfide bonds were only formed over the N-terminal ends. The size of a single SP-D polypeptide is indistinguishable from that of conglutinin. However, it migrates as a much smaller protein (approximately 160 kDa) than conglutinin on SDS-PAGE under non-reducing conditions [47]. This implies that SP-D is oligomerized using different disulfide linkages from conglutinin. While conglutinin is likely to consist of 12

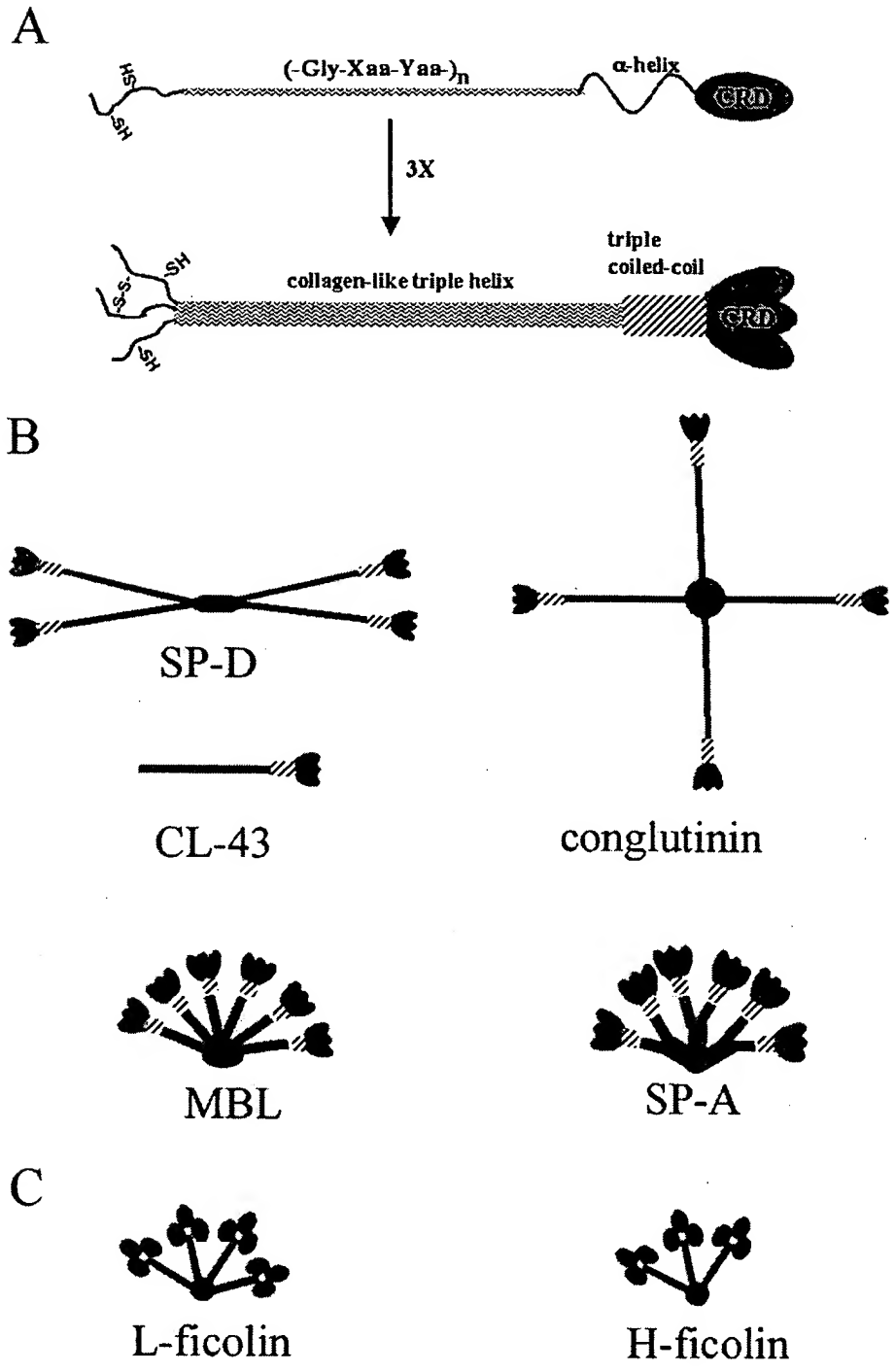

Fig. 1. Primary and tertiary structures of collectins and ficolins. The domain organization of the collectin polypeptides is illustrated. The N-terminal segment containing cysteine residues (–SH) is followed by the collagen-like, Gly–Xaa–Yaa repeating sequences, and then by a α-helical, coiled-coil sequence and a C-terminal C-type CRD (A). Three such polypeptides form a triple helix over the collagen-like region and a triple coiled-coil structure over the neck region bringing three CRDs at the C-termini together as a cluster. The N-terminal segment form inter-chain disulfide bonds leading to higher oligomeric forms (B). SP-D and conglutinin are predominantly tetramers of the triple-helical structural unit. CL-43 is only observed as monomers and SP-A is assembled into hexamers. MBL is found in a series of oligomers, that is, from monomers to hexamers and here only hexameric MBL is presented. Ficolins lack the triple coiled-coil neck region and have shorter collagen-like sequences. Therefore, ficolins are assembled into molecules with decreased dimensions compared with collectins. Trimers and tetramers have been observed for H- and L-ficolins, respectively, which may further dimerize non-covalently [10,55].

polypeptide chains assembled into four triple-helical structures that are completely disulfide-linked at the N-terminal ends, the 160-kDa SP-D molecule could represent a single trimer of three polypeptides. The SP-D molecule is also observed as a tetramer of the triple-helical structures merged at the N-terminal ends, which could be assembled from

4 × 160 kDa structures [46,47,50]. Further aggregation of the SP-D tetramers through the central knob region has also been observed [50]. SP-D is very similar to conglutinin in domain organization, size and amino acid sequence. However, conglutinin contains an additional cysteine residue in the fifth Gly–Xaa–Yaa repeat of the collagen-like region, which is not present in SP-D [15]. It is possible that this third cysteine residue in conglutinin is essential for the higher degree disulfide-linked association over the N-terminal segments including the first five Gly–Xaa–Yaa repeats. Although conglutinin has a collagen-like region largely similar to that of SP-D in length, the triple helices of conglutinin observed under electron microscope are significantly shorter than that of SP-D, that is, 37 vs. 46 nm. CL-43 has a collagen-like region that is shorter than that of conglutinin and SP-D but longer than that in MBL and SP-A. It has so far only been observed as a monomer composed of three disulfide-linked polypeptide chains [39]. As in CL-43, the N-terminal segment of SP-D also contains two cysteine residues. Therefore, it is probably the secondary structures formed by the N-terminal segment of SP-D that provide additional, non-covalent interactions between the N-terminal ends of the collagen triple-helices.

The collagen-like sequences of SP-D and CL-43 are continuous so that they form rigid and straight triple helices. The cysteine residue within the fifth Gly–Xaa–Yaa repeat of conglutinin appears likely to merge to the five Gly–Xaa–Yaa repeats into a large knob with the N-terminal segments. The collagen-like sequences in MBL and SP-A are both interrupted by irregular sequences. In MBL, the eigthth Gly–Xaa–Yaa repeat is not complete (Gly–Gln) [51]. However, the triple helices are apparently straight as viewed under the electron microscope [21]. SP-A has a more obvious interruption between the 13th and 14th Gly–Xaa–Yaa repeat, that is, Pro–Cys–Pro–Pro [52]. The electron microcopy image of SP-A shows a significant bent in the middle of the triple helices corresponding to the interrupted region [53]. The largest oligomers of human MBL and SP-A consist of six triple helices merged over their N-terminal region, and therefore, each molecule of MBL or SP-A contains 18 constituent polypeptides [21,53]. Lower oligomers, ranging between monomers and pentamers, are also consistently observed in purified MBL and these different oligomers are completely disulfide-linked [21]. However, these lower oligomers are not found in SP-A [53]. The arrangement of the disulfide bonding in SP-A is not clear. One cysteine residue is found in the N-terminal segment and a second cysteine is present in the middle of the collagen-like sequence within the Pro–Cys–Pro–Pro interruption [52]. The electron microscopy images of SP-A suggest that the six triple helices are organized into three pairs, with each pair being disulfide-linked through the cysteine residue within the Pro–Cys–Pro–Pro sequence merging the N-terminal halves of the two helices and allowing the C-terminal halves of the two helices to bend away from the central axis of the triple helix pair [53] (Fig. 1). A complete SP-A molecule can be formed from three such helix pairs that merge at the N-terminal ends partially or completely through disulfide-linkages involving the N-terminal cysteine residue. This results in an overall structure of SP-A that is indistinguishable from that of Clq [41,53]. Considering the influence of the number of cysteine residues over the N-terminal regions of the collectins on the assembly of the collectin molecules, it can be deduced that three cysteines are required to assemble collectins into completely disulfide-linked oligomers, for example, in MBL and conglutinin.

Ficolins are similarly assembled into triple helices over their collagen-like region and are also observed as disulfide-linked high molecular weight oligomers on SDS-PAGE. L-ficolin migrates with a molecular mass corresponding to 320 kDa under non-reducing conditions [10,54,55]. In the electron microscope, L-ficolin molecules are clearly observed as tetramers of the triple helices merged at their N-terminal ends [55]. The electron micrographs of H-ficolin are less clear due to the lack of top view images, but show large FBG domains and short collagen helices [13]. It has been proposed as a trimer of triple helices that can further dimerize non-covalently because it elutes as a molecule of 650 kDa by gel-filtration equivalent to approximately 18 H-ficolin polypeptides [13]. L-ficolin also undergoes further non-covalent dimerization that involves inter-molecular interactions between the FBG domains [55]. Electron microscopy images have not been obtained for M-ficolin.

4. Sugar specificity

The lectin activity of collectins is located in the C-terminal, C-type CRDs, which can be divided into two classes based on a three-residue motif in the CRDs: Glu–Pro–Asn that enables the CRD to bind mannose with a higher affinity than galactose, or Gln–Pro–Asp that makes the CRD a galactose-binding domain [56]. All collectins, except for SP-A, contain the Glu–Pro–Asn mannose-specific motif. SP-A has an Arg (in dog) or an Ala (in man) residue in place of the mannose-specific Asn residue [56]. Collectins, including SP-A, have been shown to bind to a range of sugar residues including mannose, fucose, glucose, maltose, GlcNAc and *N*-acetyl-D-mannosamine (ManNAc), although individual collectins display different preferences for certain sugar residues over the others (Table 1). MBL binds to sugars in the order of GlcNAc>D-mannose/L-fucose>ManNAc≫maltose>glucose [57]. Conglutinin displays high affinity for GlcNAc with lower affinity in the order of mannosamine>L-fucose/mannose>glucose>ManNAc/glucosamine>maltose [15]. Conglutinin is not expressed in species other than the bovidae and its closest homologue is SP-D [15]. Despite the high degree of structural similarity between SP-D and conglutinin, SP-D displays quite different sugar specificity from that of conglutinin [15]. SP-D has little affinity for GlcNAc, the best

sugar ligand for conglutinin. It binds sugars in the following order, that is, maltose>L-fucose>mannose>glucose≫glu-glucosamine, showing the highest affinity for maltose, a very weak sugar ligand for conglutinin [15]. CL-43 binds sugars in the following order, that is, mannose/ManNAc> fucose>GlcNAc>glucose and maltose>galactose>lactose≫ GalNAc, which is similar to MBL, although structurally it resembles conglutinin and SP-D [19]. Discrepancy has been reported on the affinity of SP-A for the galactose residue. By affinity chromatography, Haagsman et al. [58] showed that SP-A bound to mannose, L-fucose, galactose, and glucose. In another study, Haurum et al. [57] demonstrated binding of SP-A to a similar list of sugars, that is, Man-NAc>L-fucose, maltose>glucose>mannose, but not galactose.

The lectin activity of L- and M-ficolin, and possibly that of H-ficolin, appears to reside in the FBG domains [11,38]. L-ficolin was identified based on its affinity for immobilized yeast mannan [10]. However, L-ficolin was found to bind to cyanogen bromide-activated agarose which did not have mannan coupled to it, but had simply been inactivated with Tris [54]. All three ficolins bind to GlcNAc. L-ficolin shows no obvious affinity for sugars such as mannose, glucose, galactose and lactose. H-ficolin displays affinity for GalNAc and D-glucose as well as GlcNAc. A wider range of sugars needs to be examined to determine the sugar specificity of ficolins. A critical difference between the C-type CRD and the FBG domain in sugar binding is that Ca^{2+} is essential to the lectin activity of C-type CRDs, whereas it is not required for the lectin activity of the FBG domains of L- and M-ficolins [54]. It is not clear whether this observation is also true for H-ficolin.

5. Microbial targets

The lectin domains of collectins and ficolins undergo two grades of clustering during assembly. Primary clustering is brought about through the formation of collagen-like triple helices and, in collectins, the formation of the triple coiled-coil 'neck' structure (Fig. 1). The clustering of three CRDs in close proximity significantly increases the affinity for small sugar clusters. Further clustering occurs through the merging of multiple triple-helical structures at the opposite ends of the helices. The impact of this further clustering can be many-fold. First, it probably ensures that these molecules only bind with high affinity to dense sugar arrays, typically found on the surface of microbes, for example, fungi and bacteria. Secondly, it provides a large enough docking surface for effector molecules. Selectins function to support leukocyte rolling on endothelium for which low-affinity interaction with carbohydrate structures is essential [59]. Accordingly, selectins only contain a single C-type CRD and the receptors are not constitutively clustered. Like the collectins, the mannose receptor also recognizes microbial surface carbohydrate structures. Accordingly, each mannose receptor contains eight CRDs, which are clustered in the form of tandem repeats [60,61]. Further clustering of the CRDs can occur when multiple mannose receptors are recruited to phagocyte–microbial interfaces. Therefore, the collectins and ficolins are ideally assembled for microbial recognition and represent a unique class of soluble PRRs.

Common microbial structures that are recognized by the collectins are lipopolysaccharides (LPS) and mannan-like high-mannose structures. LPS is a major glycolipid component on the outer membrane of Gram-negative bacteria and

Table 2
Microorganisms recognized by collectins and ficolins

	Bacteria	Fungi/yeast	Viruses
MBL	*Staphylococcus aureus*, β-hemolytic group A streptococci, *Escherichia coli*, *Klebsiella* species, *Haemophilus influenzae* type b, β-hemolytic group B streptococci, *Streptococcus pneumoniae*, *Staphylococcus epidermidis*	*Saccharomyces cerevisiae*, *Aspergillus fumigatus*, *Candida albicans*, acapsular *Cryptococcus neoformans*	HIV, IAV, RSV
SP-A	*Staphylococcus aureus*, type 25 pneumococci, *Haemophilus influenzae*, *Streptococcus pneumoniae*, Group A Streptococcus, *Escherichia coli*, J5, *Mycoplasma pulmonis*, *Klebsiella pneumoniae*	*Saccharomyces cerevisiae*, *Aspergillus fumigatus*, acapsular *Cryptococcus neoformans*, *Pneumocystis carinii*	IAV, RSV, HSV-1
SP-D	*Mycobacterium tuberculosis* bacilli, Acapsular *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli*	*Saccharomyces cerevisiae*, *Aspergillus fumigatus*, *Candida albicans*, acapsular *Cryptococcus neoformans*, *Pneumocystis carinii*	IAV, RSV, rotavirus
Conglutinin		acapsular *Cryptococcus neoformans*	IAV, rotavirus
CL-43		acapsular *Cryptococcus neoformans*	rotavirus
CL-P1	*Escherichia coli*, *Staphylococcus aureus*		
L-ficolin	*Salmonella typhimurium*		

HIV, human immunodeficiency virus; IAV, influenza A virus; RSV, respiratory syncytial virus; HSV-1, herpes simplex virus type 1.

fungi are covered with rigid matrices of polysaccharides. These are ideal ligands for collectins. Collectins have been reported to bind to a wide range of microbes including bacteria, fungi and viruses [62–65]. Ficolins have also been shown to bind to the surface or LPS of bacteria that involve the lectin activities [10,13,37]. The microbes that are recognized by each individual collectin and ficolin are listed in Table 2. A number of fungal/yeast species is recognized by multiple collectins including *Saccharomyces cerevisiae*, *Aspergillus fumigatus*, *Candida albicans*, acapsular *Cryptococcus neoformans* and *Pneumocystis carinii* [65–71]. Collectins also bind selectively to several types of virus including the human immunodeficiency virus (HIV-I), influenza A virus (IAV), respiratory syncytial virus (RSV), and rotavirus [72–79] although binding of SP-D to RSV is controversial [77,78]. SP-A enhances phagocytosis of herpes simplex virus-1 (HSV-1) by alveolar macrophages though there is no evidence that SP-A binds to HSV-1 [80]. SP-D binds to several different bacteria including *Mycobacterium tuberculosis* bacilli, acapsular *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Escherichia coli* [81–84]. SP-A binds to *Staphylococcus aureus*, type 25 pneumococci, *Haemophilus influenzae*, *Streptococcus pneumoniae*, Group A Streptococcus with a preference for Gram-positive bacteria [64,85,86]. In fact, some Gram-positive bacteria are also recognized by MBL and SP-D. It is interesting to note that MBL and SP-D have been shown to bind, in addition to LPS, to PAMPs which are characteristic of Gram-positive bacteria, that is, lipoteichoic acid (LTA) and/or peptidoglycan [87,88]. The wide range of microbes that are recognized by the collectins highlights the importance of collectins in innate immunity against these pathogens. Ficolins bind to sugar structures on bacteria. H-ficolin binds to LPS derived from *Salmonella typhimurium* and *Salmonella minnesota* [13]. L-ficolin also binds to *S. typhimurium* [10]. There is no direct evidence that M-ficolin binds to microbes. However, it has been shown to promote phagocytosis of *E. coli* K-12 by monocytes [12].

6. Functional mechanisms

Binding of collectins and ficolins to microbes through the lectin domains leads to activation of multiple immunological processes such as complement activation and phagocytosis. Collectins have also been shown to modulate leukocyte chemotaxis, proliferation and differentiation. Some of these processes are not necessarily dependent on the lectin activity of these molecules.

6.1. Complement activation

The complement system is an important arm of innate immunity and is found predominantly in the blood circulation as a serine protease cascade that can be triggered by different mechanisms [89]. Complement activation on the surface of a microorganism kills microbes through lysis and then opsonizes microbial skeletons for effective phagocytosis through deposition of complement fragments. The complement cascade can be activated through three distinct pathways, that is, the classical, alternative and lectin pathways. The activation of the classical pathway requires presensitization of microbes with antibodies that are recognized by C1q. C1q is associated with the C1r and C1s serine protease proenzymes as a complex called C1 and, upon binding of C1q to antigen-bound IgG and IgM, C1r and C1s are activated which subsequently recruit and activate C4 and C2. Activated C4 and C2 form C3 convertase that recruits and activates C3 leading to the formation of membrane attack complexes and microbial lysis. The activated C3 and C4, that is, C3b and C4b, are covalently deposited on the microbial surface which are recognized by phagocytic receptors on multiple phagocytes (Fig. 2).

The alternative and lectin pathways are independent of clonal immunity. The alternative pathway is activated on the surface of certain microbes directly through the amplification of tick-over C3 activation, whereas the lectin pathway is activated after direct binding of MBL and L-ficolin to the surface sugar residues on microbes [3–7,14]. Ikeda et al. [90] first demonstrated that, like C1q, MBL could activate the complement system upon binding to yeast mannan-coated erythrocytes in a C4-dependent manner, implying the activation of the classical pathway. Independently, Ji et al. [91] identified a protein complex in mouse serum, called the 'Ra-reactive factor' which bound specifically to Ra chemotype strains of *Salmonella*, and could activate complement in a C4/C2-dependent and C1-independent manner. This protein complex includes two components: P28 and P100; P28 was later shown to be mouse MBL [92,93]. In vitro studies demonstrated that MBL could interact with and activate C1r and C1s upon binding to carbohydrate ligands [23,94]. However, a group of three C1r/C1s-like serine proteases, called MBL-associated serine proteases (MASPs), and one non-serine protease protein (MAp19), but no C1r/C1s, have been found to form complexes with MBL and are considered to be involved with MBL-mediated complement activation in vivo [95–99]. The P100 component of RaRF was also shown to be mouse MASP [93].

The three MASPs, and the MAp19, are expressed from two MASP genes, that is, MASP-1 [95], MASP-2 [96], MAp19 [98] and MASP-3 [99], with MAp19 and MASP-3 being alternatively spliced isoforms of MASP-2 and MASP-1. Recently, these MASPs have been reported to form complexes preferentially with different MBL oligomers [99]. MBL is assembled from a structural unit that consists of three identical polypeptides into trimer to hexamers of this structural unit through disulfide linkages over the N-terminal ends (Fig. 1). Differential oligomerization has not been observed in other collectins and the mechanism that leads to the different degrees of MBL oligomerization is not clear. Trimers and tetramers are consistently the predominant oligomers in purified MBL

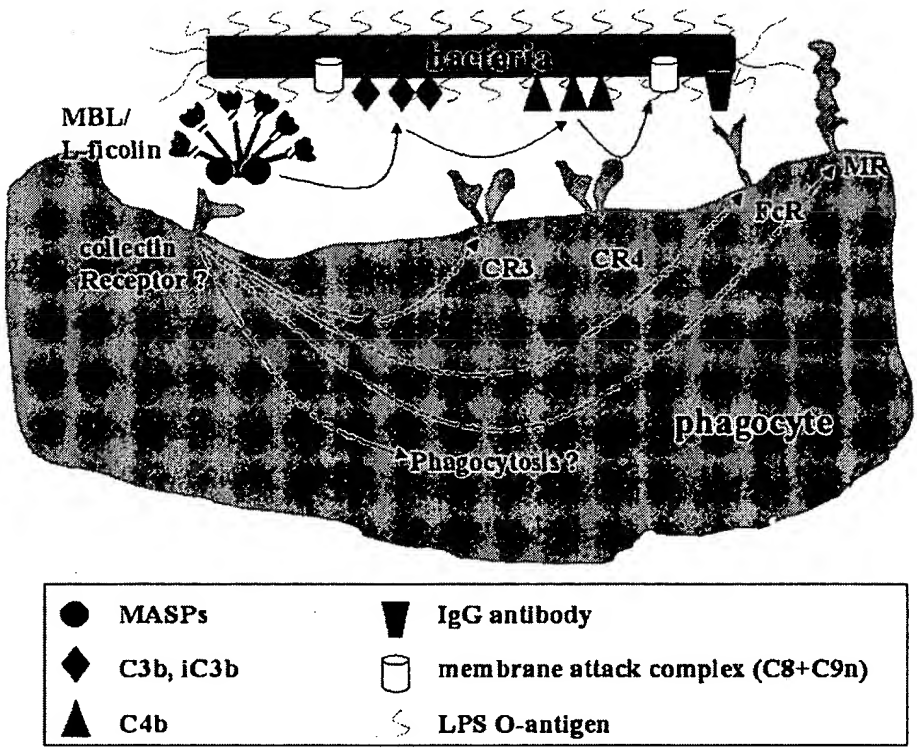

Fig. 2. Collectin-mediated microbial killing and clearance through complement activation and phagocytosis. Collectins and ficolins enhance phagocytosis either directly or, in the case of MBL and L-ficolin, indirectly through deposition of complement C3b and C4b on microbial surfaces. Phagocytes express phagocytic complement receptors CR3 and CR4. Collectins can also activate phagocytes and up-regulate phagocytosis mediated by CR3, Fc receptor (FcR) or the mannose receptor (MR) in a complement-independent manner.

(~ 80%) [23]. MASP-1 and MAp19 form a complexes with smaller MBL oligomers and then appear, via MASP-1, to activate the complement C3 directly, although the physiological significance of the low levels of C3 activation in such in vitro studies has been questioned [100]. MASP-2 forms a complex with larger MBL oligomers to activate C4 and C2 and MASP-3, which also forms a complex with the larger MBL oligomers, appears to act as an antagonist of MASP-2 activity in C4 and C2 cleavage [99]. This implies that the different MBL oligomers, in association with different MASPs, are able to activate complement along the classical and, potentially the alternative, pathway and the MBL–MASP2 complex is most closely related to the C1 complex. The association between MASP-1 and smaller MBL oligomers may involve distinct inter-molecular bonds since activated MASP-1 forms strong non-covalent interactions with MBL which cannot be interrupted by EDTA [101]. This type of interaction has not been detected between MASP-2 and MBL which is likely to be Ca^{2+}-dependent. The MASP-1 that co-purified with MBL in previous studies, which were historically considered an artificial dimer of the MBL polypeptide on SDS-PAGE under reducing conditions, may have contributed to the C1s activation observed in vitro [23,94,101]. However, the physiologically relevant in vivo targets, for MASP-1 and MASP-3, remain to be established.

The MBL–MASP pathway of complement activation is an important innate immune mechanism. Deficiency in MBL expression results in an opsonic defect, characterized by the inability of patient serum to promote microbial phagocytosis due to insufficient complement deposition on microbes [22,102]. Recently, L-ficolin has also been shown to complex with MASPs and, upon binding to *S. typhimurium*, to activate the complement system in a similar manner as MBL [103]. L-ficolin is present in serum at similar concentrations as MBL [11,102]. Therefore, the ficolin arm of the lectin pathway is potentially comparable to the MBL arm in strength. This may partially explain why a highly prevalent MBL deficiency (5–9%) was tolerated during the evolution and also raise a question in the assessment of the clinical manifestations associated with MBL deficiency. Therefore, the question can be asked—"Are both MBL and L-ficolin deficiencies required to produce a detectable complement and opsonic defect?". The difference in sugar specificity of L-ficolin from MBL means that the L-ficolin and MBL arms are complementary and may target to different, though overlapping, microbial populations. The Ca^{2+}-independent nature of ficolin binding to sugars is another property that distinguishes L-ficolin from MBL although the functional implications remain to be determined [54]. Nevertheless, although L-ficolin deficiency has not been reported, the outcome of MBL, or

potential L-ficolin, deficiency is likely to be determined by the types of invading microbes recognized by either, or both, MBL and L-ficolin.

6.2. Phagocytosis

Collectins and ficolins have been reported to promote phagocytosis of various microorganisms, either directly after binding to microbes, or indirectly through upregulation of phagocytosis mediated by other phagocytic receptors. This has been discussed in a number of review articles [2,3,6,7,14,104]. MBL and L-ficolin can indirectly opsonize microbes with complement opsonins C3b and C4b for enhanced phagocytosis without interaction with a MBL or ficolin receptor on phagocytes [14,103]. Collectins and ficolins can also function as opsonins, which require interaction of these proteins with phagocyte receptors. Such receptors have not been identified for ficolins. However, a number of putative receptors/binding proteins, for the collectins, have been identified [105,106], including calreticulin [107], C1qRp [108], SPR-210 [109], gp340 [110], CD91 [111], CR1 [112] and CD14 [113,114]. Among these receptors, calreticulin and C1qRp have been shown to bind to multiple collectins. SPR-210 and gp340 only exhibited affinity for SP-A and SP-D. Both CD91 and CR1 bind to MBL. In fact, MBL mediates phagocytosis of apoptotic cells by binding to calreticulin on apoptotic cells and CD91 on phagocytes [111]. C1qRp is a receptor that mediates immobilized collectin-induced upregulation of phagocytosis through complement and Fc receptors [108]. However, C1qRp (CD93), defined initially as a receptor on the basis of its involvement in the enhancement of phagocytosis by monocytes, appears now to be involved in adhesion events, rather than C1q-mediated phagocytosis [100]. SP-A has also been reported to promote phagocytosis of *M. tuberculosis* indirectly through upregulation of the activity of the mannose receptor but, SP-A receptor involved, remains to be characterized [115]. Soluble CD14 has recently been shown to bind to SP-A, SP-D and MBL [113,114]. CD14 is a recognition component of the LPS receptor complex that elicits inflammatory reactions upon binding to LPS [116]. The collectins may therefore regulate the expression of cytokines involved in inflammation.

SP-A and SP-D have been shown to interact with alveolar macrophages, neutrophils, peripheral blood monocytes, monocyte-derived macrophages, and bone-marrow-derived macrophages, and modulate their functions. SP-A increases intracellular Ca^{2+} and inositol triphosphate (IP_3) concentrations in alveolar macrophages. The calcium response correlates with IP_3 generation, which is necessary for SP-A-mediated phagocytosis. SP-A also stimulates chemotaxis via cell interaction involving its collagen region. It also stimulates directional actin polymerization [117]. These two effects are preceded by receptor binding and transmission of intracellular signals. SP-A can inhibit production of cytokines in response to LPS and *C. albicans* in buffy-coat cells. In contrast, it enhances production of TNF-α and colony-stimulating factor (CSF) by alveolar macrophages, and of TNF-α, IL-1α, IL-1β, IL-6 and IFN-γ by human peripheral blood mononuclear cells (PBMCs).

SP-A and SP-D also modulate the production of reactive oxygen and nitrogen species by alveolar macrophages and monocytes. SP-A enhances nitric oxide metabolite production by IFN-γ-activated alveolar macrophages, when challenged with *Mycoplasma pneumoniae*. However, SP-A inhibits nitric oxide production by IFN-γ-activated alveolar macrophages when exposed to *M. tuberculosis*, suggesting that the state of activation of phagocytic cells and the type of pathogen may affect SP-A-mediated inflammatory response. SP-A and SP-D are also known to enhance chemotactic, phagocytic and superoxidative burst properties of neutrophils [118].

6.3. Interaction of SP-A and SP-D with allergens

SP-A has been shown to bind a variety of allergenic pollens such as Lombardy poplar, Kentucky blue grass, cultivated rye and short ragweed, via its CRDs [119]. SP-A, SP-D and a recombinant fragment of human SP-D composed of homotrimeric neck and CRD regions (rfSP-D) can also bind house dust mite extract (*Dermatophagoides pteronyssinus*; Derp) in a carbohydrate-specific and Ca^{2+}-dependent manner, inhibit specific IgE binding to these glycoprotein allergens, and block allergen-induced histamine release from basophils isolated from *Derp*-sensitive patients [120]. Similarly, SP-A, SP-D and rfSP-D can also bind to the 3-week culture filtrate (3wcf) of *Aspergillus fumigatus* (Afu) as well as purified glycoprotein allergens, gp55 and gp45, inhibit the ability of *Afu*-specific IgE to bind these allergens, and block *Afu* allergen-induced histamine release from sensitized basophils isolated from ABPA patients [115]. SP-A and SP-D have been reported to reduce the proliferation of PBMCs isolated from mite-sensitive asthmatic children [121], and SP-D, in particular, has a suppressive effect on the secretions of IL-2 by PBMCs [122]. SP-A can attenuate production of IL-8 by eosinophils in a concentration-dependent manner [123]. Thus, SP-A and SP-D inhibit histamine release in the early phase of allergen provocation and suppress lymphocyte proliferation in the late phase of bronchial inflammation—the two essential steps in the development of asthmatic symptoms.

Abnormal levels of SP-A and SP-D in the lung lavage have been reported in the adult respiratory distress syndrome (ARDS) and pulmonary infections caused by influenza virus, mycoplasma and *P. carinii* in AIDS patients, hypersensitivity lung diseases and cystic fibrosis [124,125]. Asthmatics show increased amounts of SP-A in bronchiolar and alveolar lavage and SP-D in alveolar lavage as compared with those in controls [126]. Serum SP-D levels for two allergic patients have been found elevated at diagnosis which decrease following corticosteroid therapy [127]. The patients of birch pollen allergy and pulmonary alveolar

proteinosis showed a shift toward lower oligomeric forms of SP-A in comparison to healthy volunteers [128]. Depolymerisation of SP-A may lead to loss of binding affinity for carbohydrate-rich surfaces, with subsequent loss or alteration of biological function. SP-A has been shown to inhibit the LPS-induced TNF-α and IL-1 production by human alveolar macrophages in ARDS patients [129].

Recently, human SP-A, SP-D and a recombinant fragment of SP-D containing homotrimeric neck and CRD regions (rfSP-D), have been shown to dampen immunological parameters associated with allergic disorder in a murine model of allergic bronchopulmonary aspergillosis (ABPA) [130], induced by allergenic and antigenic extract of *A. fumigatus*. The murine model resembled the human disease immunologically, exhibiting high levels of specific IgG and IgE, peripheral blood and pulmonary eosinophilia, and a Th2 cytokine response. Intranasal administration of SP-A, SP-D and rfSP-D significantly lowered eosinophilia and specific antibody levels, pulmonary infiltration, reduced Th2 response and raised Th1 response. Since IgE-dependent mechanisms are important in the expansion of a Th2 immune response [131], these results suggest that SP-A and SP-D may not only suppress Th2 responses by scavenging antigens and allergens and so preventing IgE-dependent activation of eosinophils, but may well directly interact with APCs (DCs) promoting the induction of IL-12-dependent Th1 responses. A direct interaction between DC and SP-D or rfSP-D has been observed (Urban et al., unpublished data). Both molecules bind to immature human DC in a Ca^{2+}-dependent and carbohydrate-independent manner. Binding of SP-D or rfSP-D is diminished on LPS-matured DC. In concordance with these observations, immature but not mature DC express the putative SP-D receptor, gp340. SP-D has also been shown to mediate binding and uptake of *Escherichia coli* by bone-marrow-derived mouse DC and enhance antigen presentation of *E. coli* expressed proteins to T-cell hybridoma [132].

7. Conclusion

Innate immunity is important in pathogen recognition and killing, antigen processing and the initiation and modulation of adaptive immunity. PRRs are directly involved in pathogen recognition by innate immune systems and can be functionally classified into two types specialized in pathogen capturing or sensing. Phagocytic receptors such as Fc, complement and lectin receptors are primarily antigen capturing receptors that are potent in the initial killing of microbes through complement activation and/or phagocytosis. Collectins and ficolins are soluble mammalian lectins that can recognize the unique 'sugar code' on microbes [133], capture the microbes, and act either as opsonins that directly present the bound microbes to phagocytes or indirectly through further opsonization with complement proteins. Collectins recognize LPS and LTA, both are PAMPs exposed on the surface of live pathogens. In contrast, TLRs recognize a wider spectrum of microbial PAMPs including LPS and LTA but the binding sites are only effectively exposed after disintegration of microbes, for example, through complement attack or phagocytosis. There is no evidence that TLRs play major roles in pathogen capturing, for example, phagocytosis. Instead, these are pathogen-sensing receptors that sample the nature of pathogens and play a more important role in the coordination of inflammation and shaping of adaptive immunity. In this sense, the collectins are in the very front line of innate immunity.

The collectins and ficolins are found predominantly in body fluids and at the interface with the environment, for example, the surfaces of the respiratory and mucosal surfaces. Structurally, these molecules are characterized by the presence of collagen-like sequences that consistently lead to highly oligomerized mature molecules. The lectin domains in these proteins are invariably clustered at the end of triple helical collagen bundles and multiple clusters are displayed with dimensions and flexibility to maximize interactions with the unique sugar arrays over microbial surfaces. The microbe-bound collectins can then act as opsonins to enhance phagocytosis and putative collectin receptors or binding proteins identified should aid in the characterization of relevant mechanisms. Collectins may also regulate the activity of other phagocytic receptors through collectin receptor signaling. The ability of MBL and L-ficolin to activate the complement system upon binding to microbial sugar structures can lead not only to microbial killing, but also complement-dependent microbial opsonization. Therefore, the collectins and ficolins form an important network of innate immunity. The ability of SP-A and SP-D to bind to allergens and to inhibit specific IgE-mediated histamine release and to suppress lymphocyte proliferation during bronchial inflammation suggest a therapeutic role of these two collectins in asthmatic disorders.

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EXHIBIT C

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The Membrane-type Collectin CL-P1 Is a Scavenger Receptor on Vascular Endothelial Cells*

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Collectins are a family of C-type lectins that have collagen-like sequences and carbohydrate recognition domains (CRD). They are involved in host defense through their ability to bind to carbohydrate antigens of microorganisms. The scavenger receptors type A and MARCO are classical type scavenger receptors that have internal collagen-like domains. Here we describe a new scavenger receptor that is a membrane-type collectin from placenta (collectin placenta 1 (CL-P1)), which has a typical collectin collagen-like domain and a CRD. The cDNA has an insert of about 2.2 kilobases coding for a protein containing 742 amino acid residues. The deduced amino acid sequence shows that CL-P1 is a type II membrane protein, has a coiled-coil region, a collagen-like domain, and a CRD. It resembles type A scavenger receptors because the scavenger receptor cysteine-rich domain is replaced by a CRD. Northern analyses, reverse transcription-polymerase chain reaction, and immunohistochemistry show that CL-P1 is expressed in vascular endothelial cells but not in macrophages. By immunoblotting and flow cytometry CL-P1 appears to be a membrane glycoprotein of about 140 kDa in human umbilical vein or arterial endothelial cells, placental membrane extracts, and CL-P1 transfected Chinese hamster ovary cells. We found that CL-P1 can bind and phagocytose not only bacteria (*Escherichia coli* and *Staphylococcus aureus*) but also yeast (*Saccharomyces cerevisiae*). Furthermore, it reacts with oxidized low density lipoprotein (OxLDL) but not with acetylated LDL (AcLDL). These binding activities are inhibited by polyanionic ligands (polyinosinic acid, polyguanylic acid, dextran sulfate) and OxLDL but not by polycationic ligands (polyadenylic acid or polycytidylic acid), LDL, or AcLDL. These results indicate that CL-P1 might play important roles in host defenses that are different from those of soluble collectins in innate immunity.

Collectins are a family of proteins that contain at least two characteristic structures, a collagen-like region and a carbohydrate recognition domain (CRD)[1] (1). These lectins are found in vertebrates from avians to humans (2). There are four groups of collectins: the mannan-binding protein (MBP) group including MBP-A and MBP-C (3), the surfactant protein A (SP-A) group (4), the surfactant protein D (SP-D) group (5), and the newly isolated collectin liver 1 (CL-L1) (6). MBP can destroy bacteria through activation of the complement pathway (7) or opsonization via collectin receptors (8). MBP and conglutinin of the SP-D group are β-inhibitors of influenza A viruses that have hemagglutination inhibition and neutralization activities (9, 10). SP-A amplifies the phagocytosis of bacteria by macrophages (11) and opsonizes herpes simplex virus (HSV) (12). SP-D agglutinates bacteria (13) and has hemagglutination inhibition activity against influenza A virus (14). These activities indicate that collectins play an important role in innate immunity (14). In addition, the type A scavenger receptor (SR-A) also contains a collagen-like domain, which forms an oligomeric structure and binding sites (15) that have a broad specificity for ligands. The primary function of scavenger receptors is the destruction and neutralization of pathogens by endocytosis and phagocytosis. Recent knockout data show that SR-AI-deficient mice are sensitive to *Listeria* and HSV infections. Thus, it appears that scavenger receptors also have a role in innate immunity (16, 17).

Here we report the molecular cloning of a new membrane-type collectin that functions as a scavenger receptor. The cDNA for this receptor was first synthesized from placenta RNA, and the receptor is called collectin placenta 1 (CL-P1). It is present mainly in endothelial cells but is not present in monocyte-macrophage lineage cells. Surprisingly, this new collectin can bind and phagocytose bacteria and yeast as well as oxidized LDL.

EXPERIMENTAL PROCEDURES

Buffers and Media—Escherichia coli lysis buffer A for the His-Tag system consisted of 6 M guanidine hydrochloride, 0.1 M sodium phos-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB005145.

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[1] The abbreviations used are: CRD, carbohydrate recognition domain; CL-L1, collectin liver 1; MBP, mannan-binding protein; OxLDL, oxidized low density lipoprotein; AcLDL, acetylated low density lipoprotein; SP-A, surfactant protein A; SP-D, surfactant protein D; SR, scavenger receptor; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcription-polymerase chain reaction; HSV, herpes simplex virus; TBS, Tris-buffered saline; TBARS, thiobarbituric acid-reactive substances; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; HUVEC, human umbilical vein endothelial cells; HUAEC, human umbilical artery endothelial cells; BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.

phate, and 10 mM Tris, pH 8.0. Column buffers B, C, D, and E consisted of 8 M urea, 0.1 M sodium phosphate, and 10 mM Tris; the pH of each buffer was 8.0, 6.3, 5.9, and 4.5, respectively. LB medium contained 1% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto-yeast extract, and 1% (w/v) NaCl. IDG medium contained 0.4% casamino acids, 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 0.5% glucose, and 1 mM $MgCl_2$. Tris-buffered saline (TBS) consisted of 20 mM Tris-HCl and 140 mM NaCl, pH 7.4, and TBS/C was TBS containing 5 mM $CaCl_2$. Coating buffer contained 15 mM Na_2CO_3, 35 mM $NaHCO_3$, and 0.05% (w/v) NaN_3, pH 9.6.

Generation of a Probe for Screening by the Polymerase Chain Reaction (PCR)—Screening an expressed sequence tag (EST) data base for potential new collectin genes revealed a novel gene in EST clone numbers W72977 and R74387. The partial clone (I.M.A.G.E. Consortium Clone ID 34472 of W72977) from a fetal heart cDNA was purchased from ATCC and used to screen a human placenta cDNA library for full-length cDNAs by plaque hybridization. To generate a digoxigenin-DNA probe, we used the PCR. Primers amplifying the DNA probe were synthesized based on the 5′ and 3′ end nucleotide sequences of the insert in clone W72977. The primers synthesized were 5′-CAATCTGATGAGAAGGTGATG-3′ for the reverse primer and 5′-ACGAGGGGCTGGATGGGACAT-3′ for the forward primer. PCR was carried out using a PCR digoxigenin probe synthesis kit (Roche Molecular Biochemicals). The reaction mixture in 50 μl consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM each of dATP, dCTP, and dGTP, 130 mM dTTP, 70 mM digoxigenin-11-dUTP, 1.25 unit of *Taq* DNA polymerase, 1 μM each primer, and 20 ng of cDNA clone W72977. PCR was performed for 30 cycles in a TaKaRa PCR thermal cycler model 480 (Takara Shuzo Co., Ltd., Tokyo). Each cycle consisted of denaturation for 20 s at 95 °C, annealing for 20 s at 60 °C, and extension for 20 s at 72 °C. The PCR product was electrophoresed on a 1% (w/v) agarose gel (Wako Pure Chemical Industries), and then extracted from the gel using a Sephaglas BandPrep Kit (Amersham Pharmacia Biotech).

Isolation of a cDNA Encoding CL-P1 by Screening a Human Placenta cDNA Library and "Cap Site Hunting"—A phage library was screened essentially as described previously (18). In brief, ~1 × 10^6 plaque forming units of a human placenta λgt11 cDNA library (CLONTECH) were plated with *E. coli* Y1090r⁻ and incubated at 42 °C for 5 h. Nylon filters (Nytran 13N; Schleicher & Schuell Co.) were prehybridized for 1 h at 68 °C in hybri-buffer (5 × SSC, 1% blocking reagent (Roche Molecular Biochemicals), 0.1% *N*-lauroylsarcosine, and 0.02% SDS), and then hybridized for 16 h at 55 °C with a digoxigenin-labeled probe in the hybri-buffer. The filters were washed twice for 5 min at room temperature in 2 × SSC, 0.1% SDS and then twice for 15 min at 55 °C in 0.5 × SSC, 0.1% SDS. The hybridized probe was detected by incubation for 30 min at room temperature with alkaline phosphatase-conjugated anti-digoxigenin antibody (Fab) (Roche Molecular Biochemicals) diluted 1:5000. The enzyme-catalyzed color reaction was carried out using a nitro blue tetrazolium salt/5-bromo-4-chloro-3-indolyl phosphate system (Wako Pure Chemical Industries) in buffer consisting of 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 50 mM $MgC1_2$. The cDNA inserts in the positive clones were amplified using the primers described above and then directly subcloned in the pCR2.1 vector of a TA cloning kit (Invitrogen). The subclones were sequenced using an Autoread DNA sequencing kit and an A.L.F. autosequencer (Amersham Pharmacia Biotech).

To identify the sequence including the transcription start site we took the cDNA including the transcription start site from the Cap Site cDNA™ (Nippon Gene, Tokyo) of human placenta by nested PCR (6, 19). This procedure is called "cap site hunting" (19). The primer sets for the first PCR were 5′-CCGGTGGACCTTGTAGTATTG-3′ of the 1RC2 primer (Nippon Gene) and 5′-TTCTTGATGAGCTGACCATGC-3′ of the TGP1 primer that were synthesized commercially. The primer sets for the second PCR were 5′-GTACGCCACAGCGTATGATGC-3′ of the 2RC2 primer (Nippon Gene) and 5′-CATTCTTGACAAACTTCATAG-3′ of the TGP2 primer which were also synthesized commercially. The reaction mixture in 50 μl consisted of LA PCR Buffer II (Mg^{2+}-free), 2.5 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP, and dTTP (Takara Shuzo Co., Ltd.), 1 μl of Cap Site cDNA™ from human placenta, 1.25 unit of TaKaRa LA *Taq* DNA polymerase (Takara Shuzo Co., Ltd.), and 0.5 μM 1RC2 primer and PR1 primer for the first PCR and 2RC2 primer and PR2 primer for the second PCR. The first PCR was performed for 35 cycles in a TaKaRa PCR thermal cycler MP, each cycle consisting of denaturation for 20 s at 95 °C, annealing for 20 s at 60 °C, and extension for 20 s at 72 °C. The second PCR was performed for 25 cycles in the same buffer and with the same conditions using 1 μl of the first PCR products as template. After gel electrophoresis the final PCR products were extracted from the agarose gel and directly subcloned in the pT7Blue T-Vector (Novagen). The subclones were sequenced using an Autoread DNA sequencing kit and an A.L.F. autosequencer (Amersham Pharmacia Biotech).

Northern Blot and RT-PCR Analyses—Human multiple tissue Northern blot membrane was purchased from CLONTECH. It contained 2 μg of poly(A)⁺ RNAs from human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. The membranes were prehybridized at 65 °C for 3 h in a solution containing 5 × SSC, 10 × Denhardt's solution, 10 mM sodium phosphate (pH 6.5), 0.5% SDS, 50% formamide, and 0.1 mg/ml denatured salmon sperm DNA. Hybridization was performed for 18 h at 65 °C with RNA synthesized *in vitro* and labeled with digoxigenin using a PCR digoxigenin probe synthesis kit (Roche Molecular Biochemicals). The template for the DNA probe was a cDNA whole insert subcloned into pBluescriptII (Stratagene). The filters were washed twice for 5 min in 2 × SSC, 0.1% SDS at room temperature and then for 15 min in 0.1 × SSC, 0.1% SDS at 68 °C. The hybridized probe was detected as described above.

Reverse transcription (RT) was carried out using total RNAs (1 μg) from brain, heart, kidney, liver, lung, trachea, bone marrow, colon, small intestine, spleen, stomach, thymus, mammary gland, prostate, skeletal muscle, testis, uterus, cerebellum, fetal brain, fetal liver, spinal cord, placenta, adrenal gland, pancreas, salivary gland, and thyroid. The RT reaction used oligo(dT)-adaptor primers (RNA LA PCR kit (avian myeloblastosis virus) version 1.1, TaKaRa Shuzo Co., Ltd., Tokyo). The RT products were amplified in a thermal cycler (TaKaRa PCR thermal cycler MP) by 28 cycles of PCR using degenerated primer sets (0.2 μM), TaKaRa LA *Taq* polymerase (1.25 units), and RT reaction products. The primer sets for PCR were 5′-TGCCCCTGGCCCTGCAGAATG-3′ (CL-P1), 5′-CCACAGCAATGAATGGCTTT-3′ (CL-L1), 5′-ATGGTGATAGTAGCCTGGCTG-3′ (MBP), 5′-GTGGAGAGAAGGGGGAGGCTG-3′ (SP-A), and 5′-GGGACAAAGGCATTCCTGGAG-3′ (SP-D) for the reverse primers and 5′-GGAGTGTCCTTTGCCTTTGAG-3′ (CL-P1), 5′-TAGCAAATACGTAGGATGAG-3′ (CL-L1), 5′-TCAGATAGGGAACTCACAGAC-3′ (MBP), 5′-CCCTGTCCCATGGCCTAAATG-3′ (SP-A), and 5′-TCAGAACTCGCAGACCACAAG-3′ (SP-D) for the forward primers. Amplicons were separated on 1.0% agarose gels.

Lipoprotein Preparation—Human LDL was prepared from human plasma by stepwise sodium bromide density gradient centrifugation (20). All sodium bromide stock solutions contained 0.25 mM EDTA. After centrifugation, LDL was recovered from the fractions with densities of 1.09–1.063 g/cm^3. Prior to oxidation, an aliquot of LDL was passed through a 10DGR desalting column (Bio-Rad) to remove EDTA. OxLDL was prepared by the incubation of LDL (2 mg/ml) at 37 °C for 24 h with 50 μM $CuSO_4$. The reaction was stopped by the addition of 0.25 mM EDTA. The electrophoretic mobility of the OxLDL toward the anode was approximately 3 times higher than that of unmodified LDL. The OxLDL contained about approximately 50 nmol of thiobarbituric acid-reactive substances (TBARS)/mg of protein (21). The TBARS of the native LDL was about 1 nmol/mg of protein. Acetylation of LDL (AcLDL) was performed as described previously (22). Acetylation resulted in the derivatization of more than 75% of the free amino groups as determined with the trinitrobenzenesulfonic acid assay (23). Labeling of LDL, OxLDL, and AcLDL with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes) was performed as described previously (24).

Antibodies—Expression of the CRD region in CL-P1 (amino acids 590–742 of human CL-P1) in *E. coli* (pPLH3 and *E. coli* GI724) was carried out as described previously (6). The fusion protein CL-P1-CRDhis was used to produce antisera in chickens. Purification and identification of the recombinant CL-P1-CRDhis was confirmed by SDS-PAGE and immunoblotting using chicken IgY purified with an EGGstract IgY purification system (Promega). The CL-P1 antibody reacted with CL-P1-CRD but not with CL-L1-CRD, human MBP-CRD, human SP-A-CRD, or human SP-D-CRD on immunoblots (data not shown). The anti-Myc monoclonal antibody was purchased from Invitrogen (catalog No. R950–25). The expression vector (pcDNA3.1/Myc-His A vector (Invitrogen)) had two tag proteins of Myc and histidine at its C-terminal end. If the anti-Myc monoclonal antibody and chicken anti-CL-P1-CRD antibody react with the plasma membranes of living transfectants, it would indicate that the C-terminal end portion of CL-P1 may be on the surface of the cells.

Cell Culture and Isolation of a Transfected Cell Line—CHO-ldlA7 cells, kindly provided by Dr. M. Krieger (MIT), which lack functional LDL receptors, were maintained at 37 °C in Ham's F-12 medium containing 5% fetal bovine serum (25). A full-length cDNA of human CL-P1 was amplified from a human placenta cDNA library by PCR using the forward primer 5′-AATGCGGCCGCACCATGAAAGACGACTTCGCAGAG-3′ and the reverse primer 5′-GCTCTAGACCGCGGTAATGCAG-

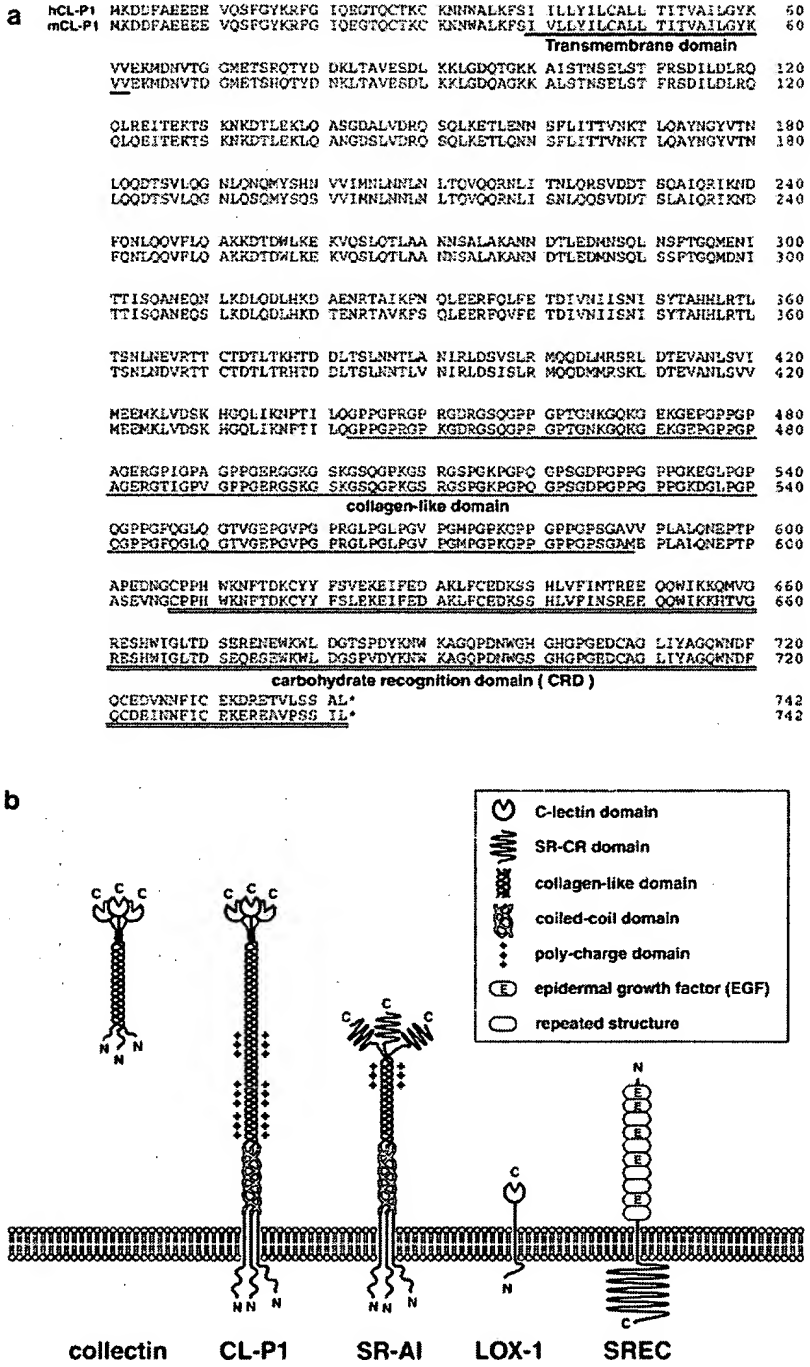

FIG. 1. *a*, the deduced amino acid sequences of human and mouse CL-P1. The amino acid residues are numbered in the N to C direction beginning with the first Met and ending with Leu. The *underlined portions* are the transmembrane domains, collagen-like domains, and CRDs. The nucleotide sequence data reported in this paper were submitted to the DDBJ, EMBL, and GenBank™ data libraries under the accession number AB005145. *b*, the structures of collectin, CL-P1, SR-AI, LOX-1, and SREC.

ATGACAGTAC-3′. The amplified human CL-P1 cDNA was subcloned into pcDNA3.1/Myc-His A vector (Invitrogen), sequenced, and transfected into CHO-ldlA7 cells using LipofectAMINE 2000 (LF2000) reagent (Life Technologies, Inc.) according to the manufacturer's protocol. To select CL-P1 positive clones, cells were cultured in Ham's F-12 medium containing 5% fetal bovine serum and 0.4 mg/ml G418 (Life Technologies, Inc.). Positive cells were detected and sorted using a FACS Vantage flow cytometer (Becton Dickinson) with anti-Myc monoclonal antibody (Invitrogen) and anti-mouse IgG-conjugated Alexa 594 (Molecular Probes). Positive clones were checked by the above method, and a stable clone (CHO/CL-P1) was established. CHO/SR-BI cells, which had been transfected with hamster SR-BI cDNA, were a gift from Dr. H. Arai (26). They were maintained at 37 °C in Ham's F-12 medium containing 10% fetal bovine serum and 0.4 mg/ml G418 (Life Technologies, Inc.).

Immunohistochemistry, Immunofluorescence Microscopy, and Western Blotting—Mice were anesthetized with 2.5% avertin and perfused through the left ventricle with 20 ml of ice-cold PBS containing 5 mM EDTA and then with 4% paraformaldehyde in PBS at 4 °C for 10 h, and hearts were collected and treated as described elsewhere (27). Specimens were dehydrated and embedded in paraffin. Ultrathin sections were stained immunohistochemically and with Mayer's hematoxylin. Immunohistochemistry was done with anti-CL-P1 antibody (chicken IgY), anti-chicken IgY conjugated with HRP (Chemicon International, Inc.), biotinylated tyramide solution, and avidin-Alexa 488 solution using the TSA™ biotin system (PerkinElmer Life Sciences). The fluorescent images were observed with an Olympus IX70–23 FL/DIC-SP and SPOT2-SP system (Olympus Optical Co. Ltd.). The transfected cells (CHO/CL-P1) were plated at a density of 3×10^4 cells/0.2 ml in 14-mm wells of 35-mm plastic culture dishes (Matsunami Glass Industries, Ltd., Japan) and cultured in Ham's F-12 medium containing 5% fetal bovine serum and 0.4 mg/ml G418. They were not fixed and directly incubated with anti-Myc murine monoclonal antibody and anti-CL-P1 chicken antibody, followed by anti-mouse IgG-conjugated Alexa 594 and anti-chicken IgY-conjugated Alexa 488 (Molecular Probes) as described previously (6). Immunofluorescent flow cytometry was performed with human umbilical vein endothelial cells (HUVEC) and human umbilical artery endothelial cells (HUAEC), both from ATCC. Cells were incu-

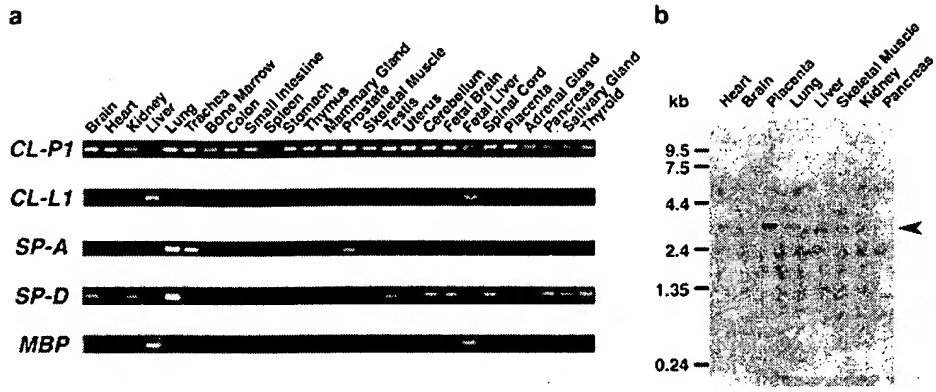

FIG. 2. **Detection of CL-P1 mRNA by RT-PCR and Northern blot analyses of poly(A)$^+$ RNAs from various human tissues.** *a*, RT-PCR analyses using total RNAs (1 μg) from brain, heart, kidney, liver, lung, trachea, bone marrow, colon, small intestine, spleen, stomach, thymus, mammary gland, prostate, skeletal muscle, testis, uterus, cerebellum, fetal brain, fetal liver, spinal cord, placenta, adrenal gland, pancreas, salivary gland, and thyroid gland. *b*, Northern blot analyses of poly(A)$^+$ RNAs (2 μg) from heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. Calculated sizes of the RNAs detected are indicated by *arrows*.

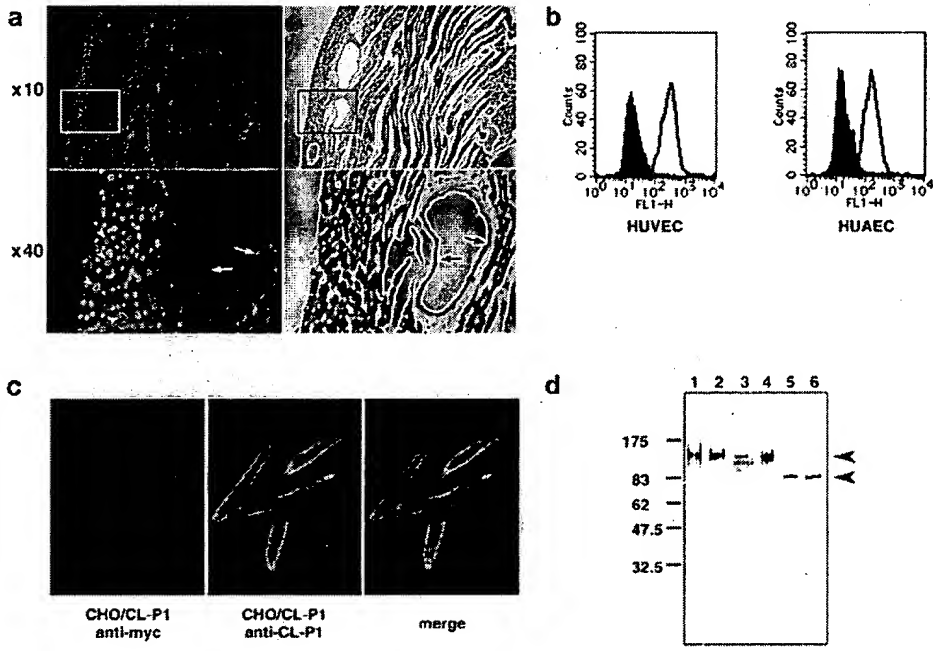

FIG. 3. **Detection of CL-P1 protein by immunohistochemistry, flow cytometry, membrane immunofluorescence, and immunoblotting.** *a*, paraformaldehyde-fixed sections of murine heart that was cut vertically were stained with chicken anti-human CL-P1-CRD antibody (*left*, × 10, × 40) and counterstained with hematoxylin eosin (*right*, × 10, × 40). The *arrowheads* indicate endothelial cells in micro and small vessels surrounding heart smooth muscle cells, and the *arrows* indicate endothelial cells in the coronary artery. *b*, flow cytometry showed membrane immunofluorescences in HUVEC and HUAEC. *c*, membrane immunofluorescence analyses showed CL-P1 on the surfaces of transfected CHO cells using anti-Myc tag and anti-CL-P1 antibodies. *d*, extracts of CHO/CL-P1 cells (*lanes 1* and *2*), HUVEC (*lane 3*), placenta (*lane 4*), deglycosylated placenta (*lane 5*), and *in vitro* transcription/translation products (*lane 6*) were subjected to SDS-PAGE, Western blotting, and probing with chicken anti-CL-P1 antibody (*lanes 1, 3, 4, 5,* and *6*) and mouse anti-Myc antibody (*lane 2*). The bound antibody was visualized with alkaline phosphatase-conjugated secondary antibody and a BCIP/NBT substrate system.

bated with anti-CL-P1 chicken antibody and anti-chicken IgY-conjugated Alexa 488 at 4 °C for 30 min and assayed with a FACS Calibur (Becton Dickinson). Appropriate cell fractions were selected using a two-dimensional display of forward scatter and side scatter. Western blotting analyses were performed using CL-P1 transfected cells, HUVEC, placental tissue membrane extracts (BioChain Institute, Inc., CA) without or with de-glycosylation (Enzymatic Deglycosylation kit, Bio-Rad), and *in vitro* transcription and translation products of CL-P1 cDNA. *In vitro* transcription/translation was performed with the TNT T7 Quick Coupled transcription translation system (Promega). All cell surfaces were biotinylated with 0.5 mg/ml sulfo-NHS-LC-biotin (Pierce). The cells were lysed with SDS-sample lysis buffer as described previously (28), and fractions were collected after incubation with anti-biotin-agarose (Sigma). Equal concentrations of protein (20 μg), cell lysates, and synthesized protein solutions were subjected to SDS-PAGE under reducing conditions, followed by electroblotting onto BioBlot-NC membranes (Corning Costar Corp.). Membranes were incubated with anti-CL-P1 chicken antibody or anti-Myc murine monoclonal antibody and alkaline phosphatase-conjugated goat anti-chicken IgY (Chemicon International, Inc.) or alkaline phosphatase-conjugated goat anti-mouse IgG (Chemicon International, Inc.). Bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BCIP/NBT, Kirkegaard and Perry Laboratories) as described previously (6).

Analysis of Lipoprotein Binding—CHO/CL-P1, CHO/SR-BI, and CHO-ldlA7 cells were plated at densities of 3 × 10^4 cells/0.2 ml in 14-mm wells of 35-mm plastic culture dishes and cultured in Ham's F-12 medium containing 5% fetal bovine serum with or without 0.4 mg/ml G418. Cells were incubated at 4 °C for 30 min with DiI-OxLDL, DiI-AcLDL, and DiI-LDL. Fluorescent images were observed with the Olympus IX70–23 FL/DIC-SP and SPOT2-SP system (Olympus Optical Co. Ltd.). CHO/CL-P1 cells were incubated at 4 °C for 2 h with 5 μg/ml DiI-OxLDL in the presence at 200 μg/ml of LDL, AcLDL, and OxLDL24, 10 μg/ml dextran sulfate, polycationic ligands (poly(A), poly(C)), and polyanionic ligands (poly(G), poly(I)) (29). To quantify the amount of DiI-OxLDL, cells were washed and then fixed with PBS containing 4% paraformaldehyde, pH 7.4, treated with 1 drop of SlowFade antifade

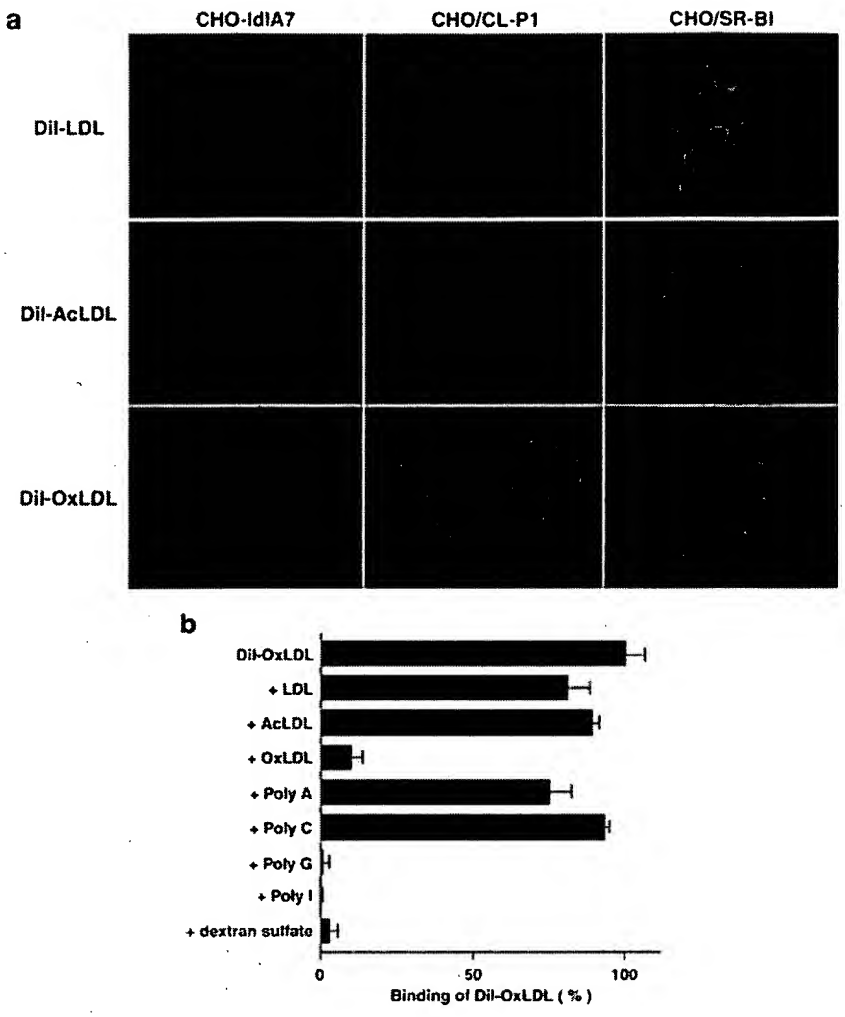

FIG. 4. **Binding of native LDL and modified LDLs to CHO/CL-P1, CHO/SR-BI, and CHO-ldlA7 cells.** *a*, DiI-LDL, DiI-AcLDL, and DiI-OxLDL were incubated at 4 °C for 30 min with CHO-ldlA7, CHO/CL-P1, and CHO/SR-BI cells. CHO/SR-BI cells were used as a positive control, and CHO-ldlA7 cells were used as a negative control. *b*, the binding of DiI-OxLDL was inhibited by poly(I,G), Ox-LDL, and dextran sulfate but not by poly(A,C), AcLDL, or native LDL. *Bars* indicate standard deviations.

reagent (Molecular Probes), mounted, and sealed. The fluorescent images were observed with the same system as above. Fluorescence intensity was quantified using IPLab imaging software (Scanalytics, Inc.).

Analysis of Microorganism Binding—CHO/CL-P1 cells were incubated at 4 °C for 2 h with 1 μg/ml *E. coli* (K12 strain) BioParticles conjugated with Texas Red (Molecular Probes), *Staphylococcus aureus* BioParticles conjugated with tetramethylrhodamine (Molecular Probes), or zymosan A (*Saccharomyces cerevisiae*) BioParticles conjugated with Texas Red (Molecular Probes). After binding, cells were fixed at room temperature for 20 min with 4% paraformaldehyde in PBS and stained with anti-Myc monoclonal antibody and anti-mouse IgG-conjugated Alexa 488. Fluorescent images were observed with the system described above. The uptake assay using *S. cerevisiae* BioParticles conjugated with Texas Red (Molecular Probes) was performed at 37 °C overnight under 5% CO_2. After the same staining, phagocytosed bioparticles were observed under a confocal laser-scanning microscope LSM510 (Carl Zeiss Co. Ltd.).

RESULTS AND DISCUSSION

Molecular Cloning of the CL-P1 Gene—We screened DNA data bases to identify novel members of the collectin family and identified a cDNA fragment from human EST data bases that showed carboxyl-terminal sequence homology with the collectins. The EST clone W72977 from a fetal heart cDNA library was used to screen a human placenta cDNA library, and positive clones were isolated. In addition, "Cap-site hunting" (19) was performed to determine the complete 5′-terminal sequence including the transcription start site of a new collectin mRNA. Restriction mapping and sequencing of the clones revealed that they contained an open reading frame of 2226 base pairs encoding a sequence of 742 amino acids (Fig. 1*a*). The deduced amino acid sequence revealed a collectin structure consisting of a collagen-like region and a CRD. This new collectin, designated collectin placenta 1 (CL-P1), has an intracytoplasmic domain, a transmembrane domain with a coiled-coil region, a collagen domain, and a CRD (Fig. 1*b*). At the amino acid level the cloned mouse CL-P1 has high sequence identity (92%) and the same length and same domain sizes as human CL-P1 (Fig. 1*a*). The homology between human and mouse CL-P1 is the highest among the collectins. The collagen domain had the highest homology and has 49 more Gly-*X*-*Y* cycles than SR-AI (15). CL-P1 has three polycationic regions in a collagen domain that contain basic amino acids (arginine or lysine). These amino acid sequences are almost identical to those in human and mouse CL-P1. CL-P1 has a C-type lectin consisting of six cysteine residues, which is highly homologous to the CRDs in macrophage lectin 2 and the asialoglycoprotein receptor (1). Its ligand specificity is of the galactose type (Gln-Pro-Asp), which is different from the mannose and glucose types (Glu-Pro-Asn) (30). The whole structure of CL-P1 resembles that of SR-AI (Fig. 1*b*). The structures of other SRs, LOX-1 (31) and SREC (29), expressed in endothelial cells are completely different from those of SR-AI and CL-P1. SR-AI and CL-P1 can form oligomeric structures due to their collagen-like regions and coiled-coil structures. The polycharge islands in the collagen-polymer structure form a strong binding site for negatively charged substances. An endocytosis motif (Tyr-Lys-Arg-Phe) (32), like in the asialoglycoprotein receptor, is present in the intracytoplasmic domain.

Localization of CL-P1 in Tissues and Cells—RT-PCR analy-

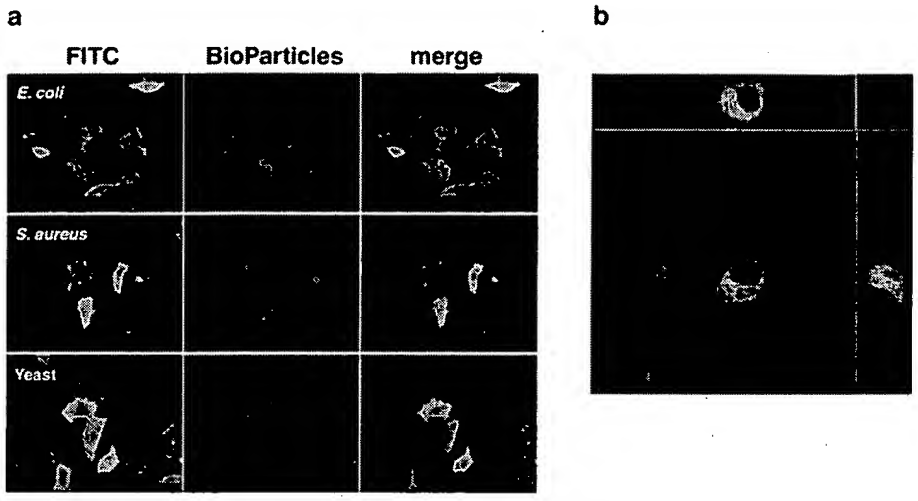

FIG. 5. **Binding of microbes to CHO/CL-P1 cells.** *a*, photographs of CL-P1 expression and microbe binding. CHO/CL-P1 cells were stained with anti-Myc antibody and anti-mouse IgG conjugated with Alexa Fluor™488. BioParticles of *E. coli*, *S. aureus*, and yeast (*S. cerevisiae*) conjugated with Texas Red or tetramethylrhodamine were used. *b*, the uptake of *S. cerevisiae* BioParticles by CHO/CL-P1 cells was performed at 37 °C overnight under 5% CO_2. After the same staining as in *a*, phagocytosed bioparticles were observed under a confocal laser scanning microscope.

ses showed that most tissues express CL-P1 mRNA, in contrast to CL-L1, MBP, SP-A, and SP-D mRNAs (Fig. 2*a*). Northern blot analyses showed a major band of about 3.2 kilobases in placenta, heart, and lung (Fig. 2*b*). Immunohistochemical analysis showed that CL-P1 is localized in murine vascular endothelial cells in the heart (Fig. 3*a*). We also found expression of CL-P1 protein in most vascular endothelial cells in all murine vessels and human heart sections (data not shown). This distribution of CL-P1 protein is consistent with the expression of CL-P1 mRNA in vascular-rich tissues. Macrophages, monocytes, and hepatic Kupffer cells did not express CL-P1 or CL-P1 mRNA (data not shown). The expression of CL-P1 in HUVEC and HUAEC was shown by flow cytometry (Fig. 3*b*). However, THP-1, U937, and HL-60 treated with lipopolysaccharide were negative in the above analyses (data not shown). A study of the expression of CL-P1 cDNA in CHO cells showed that this new collectin is a type II membrane protein because it was detected by anti-C-terminal tag antibody (anti-Myc monoclonal antibody) and anti-CRD antibody (CRD at the C-terminal end) (Fig. 3*c*). The non-transfected cells were not stained by two antibodies, respectively (data not shown). It was found that CL-P1 has an approximate molecular mass of 140 kDa in CHO/CL-P1, HUVEC, and placenta membrane extracts using anti-Myc and anti-CL-P1 antibodies (Fig. 3*d*). Deglycosylated CL-P1 produced by an *in vitro* transcription/translation system has a mass of 90 kDa, which matches the calculated molecular mass (Fig. 3d). CL-P1 has several *N*-glycosylation sites in its coiled-coil region. CL-P1 has an oligomeric structure due to its collagen-like and coiled-coil helical domains. Its molecular mass is very high under non-reducing conditions, and the truncated form of CL-P1, lacking a transmembrane domain, is a trimer of about 300 kDa as determined by gel filtration chromatography (data not shown).

Functional Analyses of CL-P1—Modified LDLs and native LDL were incubated with CHO/CL-P1, CHO/SR-BI, and CHO-ldlA7 cells. OxLDL bound to CHO/CL-P1 cells but Ac-LDL and LDL did not (Fig. 4*a*). CHO/SR-BI cells could bind all LDLs (OxLDL, AcLDL, and LDL), but CHO-ldlA7 cells did not bind any of them. The binding of DiI-OxLDL to CHO/CL-P1 cells was inhibited by negative polycharged substances (poly(I,G) and dextran sulfate) and by OxLDL but not by LDL or AcLDL (Fig. 4*b*). The TBARS of the OxLDL was about 50 nmol/mg. Another OxLDL receptor, LOX-1, showed specific binding of mildly oxidized LDL (TBARS about 10 nmol/mg) (33).

Microbes also bound to CHO/CL-P1 cells (Fig. 5). A previous study showed that *E. coli* and *S. aureus*, but not yeast, bound to MARCO (34), which is one of the SR-As. CHO/CL-P1 cells bound yeast as well as *E. coli* and *S. aureus*. The non-transfected CHO cells did not bind any of microbes (data not shown). Overnight incubation revealed that yeasts were endocytosed and digested (Fig. 5*b*). These binding activities were also inhibited by polyanionic ligands (dextran sulfate and poly(I,G)) (data not shown). Recently, it was found that SR-AI knockout mice have increased fatality from HSV and *Listeria* infections (16, 17). Here we show that a scavenger receptor may play a role in innate immunity. CL-P1 is a member of the collectin family, which is considered to play significant roles in innate immunity. Classical collectins are soluble, but CL-P1 is membrane-bound. CL-P1 might bind and control not only bacteria and yeasts but also modified LDLs in the vascular space. The collagen-like domains in human and mouse CL-P1, which have the highest identity (96%) described to date, may play the most important role in these biological functions. A detailed examination of the active binding sites is needed.

Here we identified a new collectin, CL-P1, which may have a novel function in the process of atherogenesis as well as a role in protecting against bacterial and yeast pathogens.

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